DOCTOR OF PHILOSOPHY

Arsenic, antimony and visceral leishmaniasis

Perry, Meghan Rose

Award date:
2014

Arsenic, antimony and visceral leishmaniasis

Meghan Rose Perry

PhD Thesis

July 2014

Supervisor:

Professor Alan H. Fairlamb

University of Dundee

List of figures

Chapter 1: Introduction **Facing page**

Chapter 2: Methods **Facing page**

Chapter 3: Results **Facing page**

List of tables

List of abbreviations

AAS	Atomic absorption spectrometry
AMB	Amphotericin B
APML	Acute promyelocytic leukaemia
AQP	Aquaglyceroporin
ATO	Arsenic trioxide
AS3MT	Arsenic (III) methyl transferase
ATSDR	Agency for Toxic Substances and Disease Registry
C	Complement
CL	Cutaneous leishmaniasis
DALY	Disability adjusted life year
DDT	Dichlorodiphenyltrichloroethane
DMA	Dimethylarsinic acid
ECG	Electrocardiogram
ELISA	Enzyme linked immunosorbent assay
FIND	Foundation for Innovative Diagnostics
γ-GCS	Gamma-glutamyl cysteine synthetase
GSH	Glutathione
HAT	Human african trypanosomiasis
HIV	Human immunodeficiency virus
HR	Hazard ratio
HRP	Horse radish peroxidase
i.p.	Intraperitoneal
iAs	Inorganic arsenic
ICP-MS	Inductively coupled plasma mass spectrometry
IDA	International Dispensary Association
IFAT	Immunofluoresence antibody test
IFN	Interferon
IL	Interleukin
IM	Intramuscular
IRS	Indoor residual spraying

IV	Intravenous
LAMP	Loop mediated isothermal amplification
*Lm*ACR2	*Leishmania* antimony reductase 2
LPG	Lipophosphoglycan
MA	Meglumine antimonite
MCL	Mucocutaneous leishmaniasis
MMA	Monomethlyarsenic acid
MRSA	Multi resistant staphylococcus aureus
Mya	Million years ago
NTD	Neglected tropical diseases
ODC	Ornithine decarboxylase
OR	Odds ratio
PBMC	Peripheral blood monocytes
PKDL	Post kala azar dermal leishmaniasis
PMA	Phorbol 12-myristate 13-acetate
PPG	Proteophosphoglycan
ppm	Parts per million
s.c.	Subcutaneous
SOES	School for Environmenal Studies
SSG	Sodium stibogluconate
TDR1	Thiol dependent reductase 1
TNF-α	Tumour necrosis factor – alpha
T[SH]2	Trypanothione
TB	Tuberculosis
Th	T-cell helper
TR	Trypanothione reductase
UNICEF	United Nations Children's Fund
VL	Visceral leishmaniasis
WHO	World Health Organisation

Acknowledgements

This work would not have been achieved without the incredible support of many teams of people.

First of all I would like to thank Alan Fairlamb for his guidance, support, patience and sagacity through this project. Thank you to Susan Wyllie for invaluable practical help and useful discussions. The whole AHF group and BCDD have been very helpful but I would particularly like to mention Deuan Jones, Laste Stojanovski, Frederick Simeons and Manu de Rycker. Thank you to Martin Kierans for his help with the electron microscopy.

I would like to thank my collaborators, Andrea Raab and Joerg Feldmann in Aberdeen for the arsenic quantitation and helpful advice.

The field work part of this project has been guided by Marleen Boelaert and Albert Picado – I thank them for guidance and inspiration in relation to public health. Thank you to Joris Menten for help with the statistics and to George Geddes for his help with the mapping. Marleen's team Epco Hasker and Bart Ostyn have been a great source of knowledge and humour.

I spent almost a year in total living in India during this project. Thank you to my hosts Shyam Sundar and Vijay Kumar Prajapati for incredible support, cultural and practical guidance. The following people also helped me so much during my time in India: Madhukar Rai, Jaya Chakravarty, Smriti Sharma, Rudra Pratap Singh, Arjay Singh, Ashish Srivastava, Dipankar Chakraborti, the team at KAMRC and Shyam Sundar's lab members.

All my family and friends have been an unfailing support but I would particularly like to thank Tim Vincent-Smith, who has become both a father and a husband during this project, and my son Oliver, who has been an excellent distraction.

Thank you to Sara Marshall, the director of the Wellcome PhD Programme here in Dundee, who has been informative and helpful in every decision I have made and thank you to the Wellcome Trust for funding.

Declaration

I hereby certify that this thesis is of my own composition and is based on the results of my own work, carried out under the supervision of Professor Alan H. Fairlamb. Work other than my own is specifically stated in the text by reference to the relevant researchers or to their publications. I have consulted all references cited herein, unless otherwise stated. No part of this thesis has been previously submitted for a higher degree.

Meghan Rose Perry

I certify that Meghan Rose Perry has performed the research described in this thesis under my supervision and has fulfilled the conditions of the relevant ordinance and regulations of the University of Dundee, and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

Professor Alan H. Fairlamb

Wellcome Principal Research Fellow

Abstract

In Bihar state, India, the cure rate of antimonial compounds in the treatment of visceral leishmaniasis (VL) has declined from over 85% to less than 50%. This has been attributed to prolonged, widespread misuse of antimonials within the Indian private healthcare system. An alternative resistance hypothesis is that exposure to arsenic in drinking water in this region has resulted in antimony-resistant *Leishmania* parasites.

Leishmania donovani were serially passaged in mice exposed to environmentally-relevant levels of arsenic in drinking water. Arsenic accumulation in organs of these mice was proportional to exposure. After five passages, isolated parasites were refractory to Sb^V in drug sensitivity assays. Treatment of infected mice with Sb^V confirmed that these parasites retained resistance *in vivo*, supporting this hypothesis.

A retrospective field study on a cohort of antimony treated VL patients was performed in an arsenic contaminated area of Bihar to evaluate the presence of an increased risk of treatment failure and death in those exposed to arsenic. It demonstrated a significant increased risk of death from VL in arsenic exposed patients but did not indicate a significant relationship between arsenic exposure and antimonial treatment failure.

Collectively these data suggest that it is biochemically possible that arsenic contamination may have contributed to the development of antimonial resistance in Bihar although issues of underpower and the retrospective nature of our epidemiological study made it difficult to conclusively demonstrate this. Further research in to the relationships between arsenic exposure and antimonial treatment failure and death in the leishmaniases is warranted.

Chapter 1- Introduction

1.1 Leishmania

1.1.1 Kinetoplastids

The order Kinetoplastidia contains eukaryotic parasites that include important human pathogens within the *Leishmania* and *Trypanosoma* species (Stuart *et al*., 2008). They are uni-flagellated protozoa which are characterised by the presence of mitochondrial DNA contained in a unique organelle called the kinetoplast, located near the base of the flagellum. The species *Leishmania, Trypanosoma cruzi and Trypanosoma brucei*, although sharing many similar cellular biology features, cause the distinctly different diseases of the leishmaniases (Section 1.1.2), Chagas disease and human African trypanosomiasis (HAT, also called African sleeping sickness), respectively. They all rely on invertebrate transmission between human (and in some regions mammalian hosts) in the form of the sand fly, tsetse fly and triatomine bug. The diseases affect the poorest of the poor (Boelaert *et al*., 2009) and are associated with poor housing and population displacement.

The leishmaniases, HAT and Chagas Disease are part of a group of 17 'Neglected Tropical Diseases' (NTD), classified by the World Health Organisation (WHO), that pose a significant barrier to socioeconomic development and poverty reduction in low income countries. NTDs are endemic in 149 countries and affect more than 1 billion people worldwide. In 2012, the kinetoplastid diseases were highlighted in the WHO Implementation Road Map[1] for intensified disease management intervention as they lack simple tools for control and current treatments have unacceptable toxicity profiles as well as many regional issues with efficacy. The most dramatic example of this is the necessity for continued use of the arsenical based drug melarsoprol, for the treatment of the neurological phase of HAT caused by *T.rhodesiense*, which causes a reactive arsenic

[1] http://www.who.int/neglected_diseases/NTD_RoadMap_2012_Fullversion.pdf

Table 1.1 *Leishmania* species and their sand fly vectors

Each sand fly tends to transmit one strain of parasite and each parasite tends to lead to one disease phenotype. *L. = Leishmania P. =Phlebotomus.* Adapted from (Bates *et al.*, 2007; Lukes *et al.*, 2007*).*

***Leishmania (L.)* Species**	**Disease in Humans**	**Transmission**	**Mammalian host**	**Vector**	**Geography**
L. major	Old world CL (oriental sore)	Rural zoonotic	Gerbil, sand rat	*P.papatasi, P.dubosqi, P.salehi*	Central and West Asia, North Africa, Sahel of Africa, Central and West Africa
L. tropica	Old world CL (oriental sore)	Urban anthroponotic	Humans, rock hyraxes	*P. sergenti*	Central and West Asia, North Africa
L. aethiopica	Old world diffuse CL	Rural zoonotic	Rock hyraxes	*P. longipes, P. pedifer*	Ethiopia, Kenya
L. donovani	VL	Epidemic anthroponotic	Humans	*P. argentipes, P. orientalis, P. martini*	Indian subcontinent, East Africa
L. infantum	VL (infantile)	Zoonotic, peridomestic	Domestic dog	*P. ariasi, P. perniciosus*	Mediterranean basin, Central and West Asia
L. infantum (syn Chagasi)	VL (infantile)	Zoonotic, peridomestic	Domestic dog	*Lutzomyia longipalpis*	Central and South America
L. Mexicana	New world CL (chiclero's ulcer)	Sylvatic zoonotic	Forest rodents	*Lutzomyia olmeca olmeca*	Central America
L. amazonenesis	New world CL	Sylvatic zoonotic	Forest rodents	*Lutzomyia flaviscutellata*	South America
L. (Viannia) braziliensis	New world CL and MCL (espundia)	Sylvatic zoonotic	Forest rodents	*Lutzomyia wellcomei. Lutzomyia complexua, Lutzomyia carrerai*	Central and south america
L. (Viannia) peruviana	New world CL (uta)	Upland zoonotic	Upland zoonotic	*Lutzomyia peruensis, Lutzomyia verrucarum*	Peru
L. (Viannia) guyanensis	New world CL, metastatic (pian-bois)	Sylvatic zoonotic	Sloth, anteater	*Lutzomyia umbratilis*	South America
L. (Viannia) panamensis	New world CL	Sylvatic zoonotic	Sloth	*Lutzomyia trapidoi*	Central America

Figure 1.1 Human diseases caused by the *Leishmania* parasite

Panel A shows the raised pearly edge lesion of cutaneous leishmaniasis (source CDC) Panel B depicts destruction of the nasal septum from mucocutaneous leishmaniasis (source CDC) Panel C shows profound weight loss and the massive hepatosplenomegaly found in advaced visceral leishmanaisis (source WHO) Panel D depicts diffuse depigmented macules of Post Kala Azar Dermal Leishmaniasis (Source: author's own)

encephalopathy in up to 10 % of patients that is often fatal (Pépin *et al*., 1995) (Kennedy, 2013). The Implementation Road Map has optimistic goals for 2020 of success in regional elimination programmes for the Leishmaniases and Chagas disease and global elimination of HAT. There remain many issues to be overcome for this to be achieved.

1.1.2 Leishmania epidemiology

There are 30 species of *Leishmania* that infect mammals and 21 of these cause human disease (Herwaldt, 1999) collectively named the leishmaniases. Features of the most important species with their disease phenotype and vector are summarised in Table 1.1 (Bates, 2007). These diseases are cumulatively thought to be responsible for the ninth largest infectious disease burden worldwide and together account for the loss of 2.1 million Disability Adjusted Life Years (DALYs[2]) (Stuart *et al*., 2008). The human diseases caused are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL, also called kala azar). Cutaneous leishmaniasis is a skin sore, caused by a range of *Leishmania* species (Table 1.1), which occurs, either at the site of the bite of the sand fly or following metastasis to a distant cutaneous location, and can take the form of a papule but more classically is an ulcer with a raised edge (Figure 1.1, panel A) (Chappuis *et al*., 2007). CL lesions often regress spontaneously without treatment but in endemic countries CL is a major cause of morbidity and permanent scarring (Llanos-Cuentas *et al*., 2008). Infection with the species *L.braziliensis* can lead to the dreaded complication of the development of mucocutaneous lesions where the parasite spreads to mucosal surfaces of the upper airways and can cause dramatic facial disfigurement (Figure 1.1, panel B)(Chappuis *et al*., 2007).

[2] Each disease is given a disability weight. A DALY is calculated from the sum of the years of life lost and years lived with disability (Arnesen and Nord, 1999).

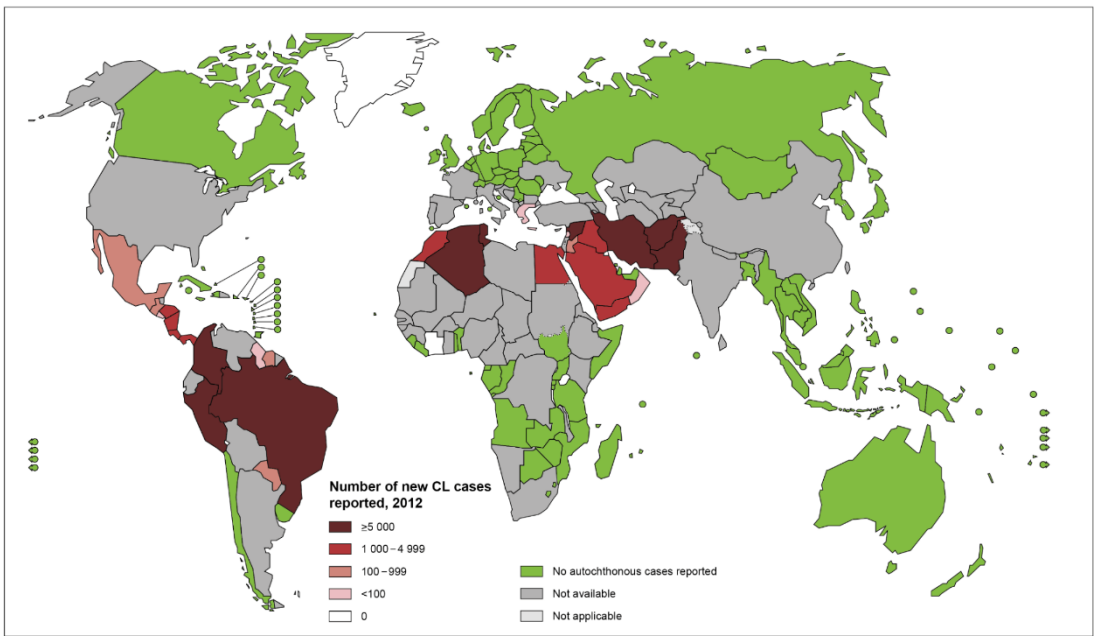


Figure 1.2 Status of endemicity of cutaneous leishmaniasis, worldwide, 2012.

Source: WHO

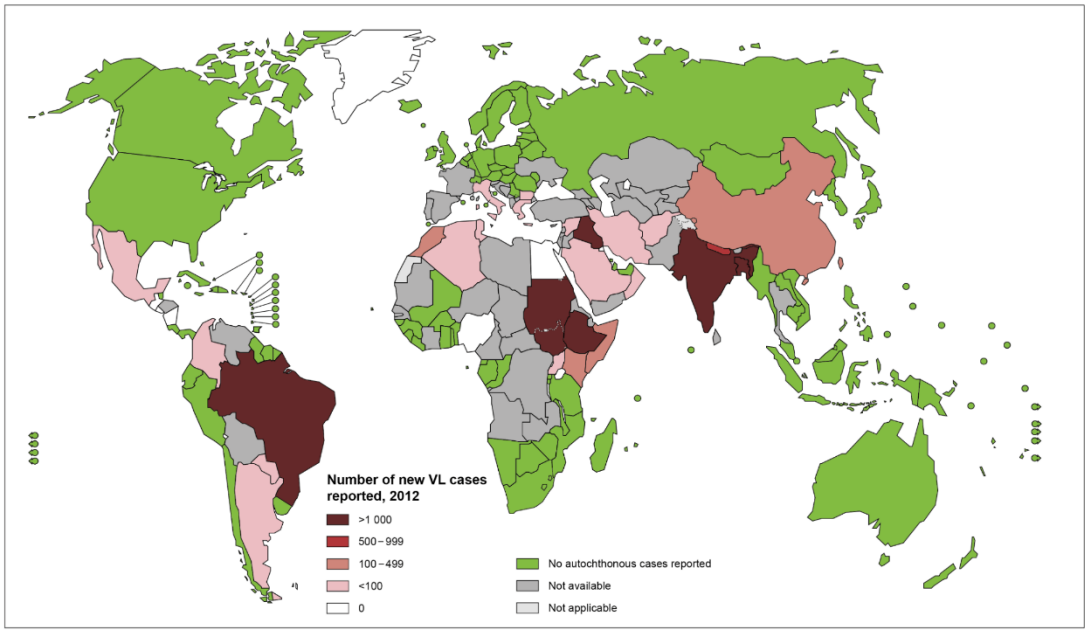


Figure 1.3 Status of endemicity of visceral leishmaniasis, worldwide, 2012.

Source: WHO

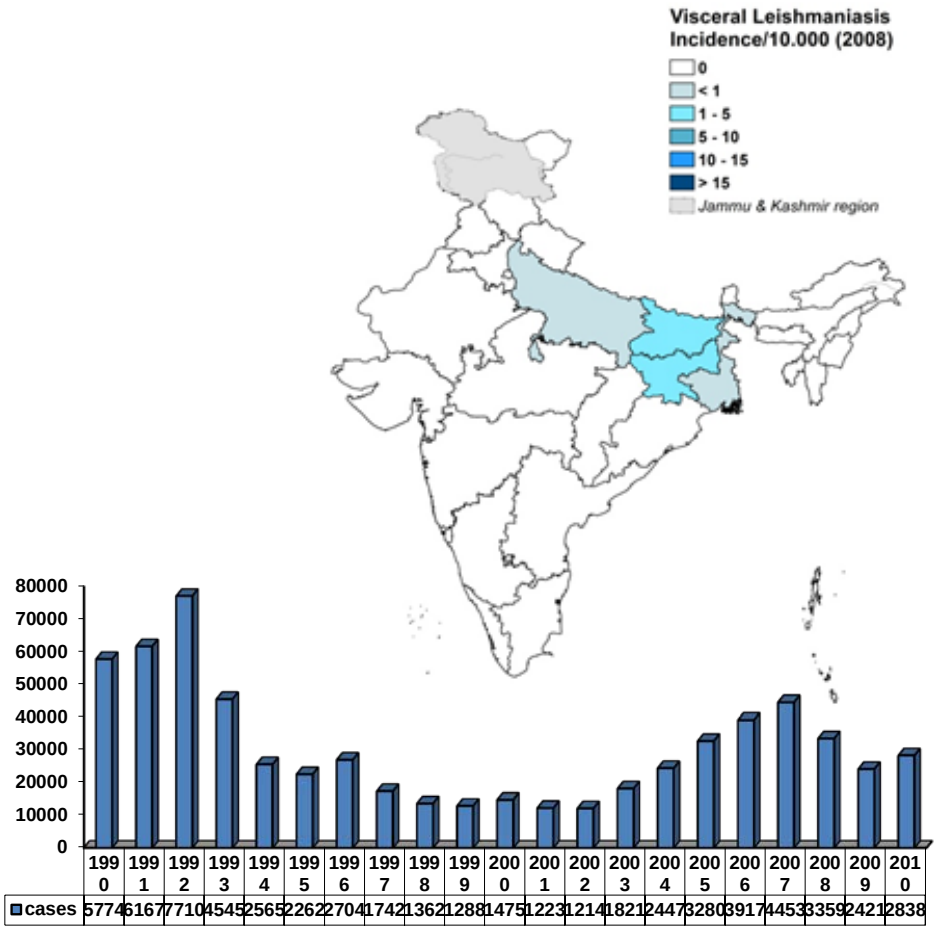


Figure 1.4 Estimates of incidence of visceral leishmaniasis India

Incidence is calculated using input from national and international experts, underreporting rates and disease figures reported to the government. Source: (Alvar *et al*., 2012)

Visceral leishmaniasis is a progressive systemic illness caused by the species *L.infantum* and *L.donovani* (Section 1.1.3) (Figure 1.1, panel C) which is fatal without treatment. The sequelae, in a proportion of treated patients is the diffuse skin condition post kala azar dermal leishmaniasis (PKDL) (Figure 1.1, panel D) (Chappuis *et al*., 2007). This study focusses mainly on visceral leishmaniasis.

There are serious issues relating to underreporting of the leishmaniases in certain regions (Mondal *et al*., 2009) but their global incidence was recently estimated at 0.7 to 1.2 million per annum for CL (including MCL) and 0.2 to 0.4 million per annum for VL (Alvar *et al*., 2012). Approximately three quarters of CL cases are found in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru (Figure 1.2). Ninety percent of mucocutaneous leishmaniasis cases occur in Bolivia, Brazil and Peru[3]. The annual reported mortality rate for VL is estimated to be 10% with between 20 and 40,000 deaths per year (Alvar *et al*., 2012). Ninety percent of VL cases are in 6 countries India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Figure 1.3). In India, the area on which this study is focussed, the VL incidence is estimated to be up to 282,800 cases per annum (Figure 1.4) (Alvar *et al*., 2012). The level of under-reporting can be up to a factor of 4.17 (Singh *et al*., 2010) (Figure 1.4).

1.1.3 Leishmania history

The *Leishmania* parasite is thought to be descended from a protist parasite *Shuiyousphaeridium spp.* that has a fossil record from 1600 million years ago (Mya)(Tuon *et al*., 2008). Kinetoplastidia lack a fossil record dating this far but fossil records of their close relatives the euglenids suggest their appearance around 450 Mya with a probable primitive water dwelling animal as host. Flying insects appeared at 300 Mya and the

[3] www.who.int/leishmaniasis/en/

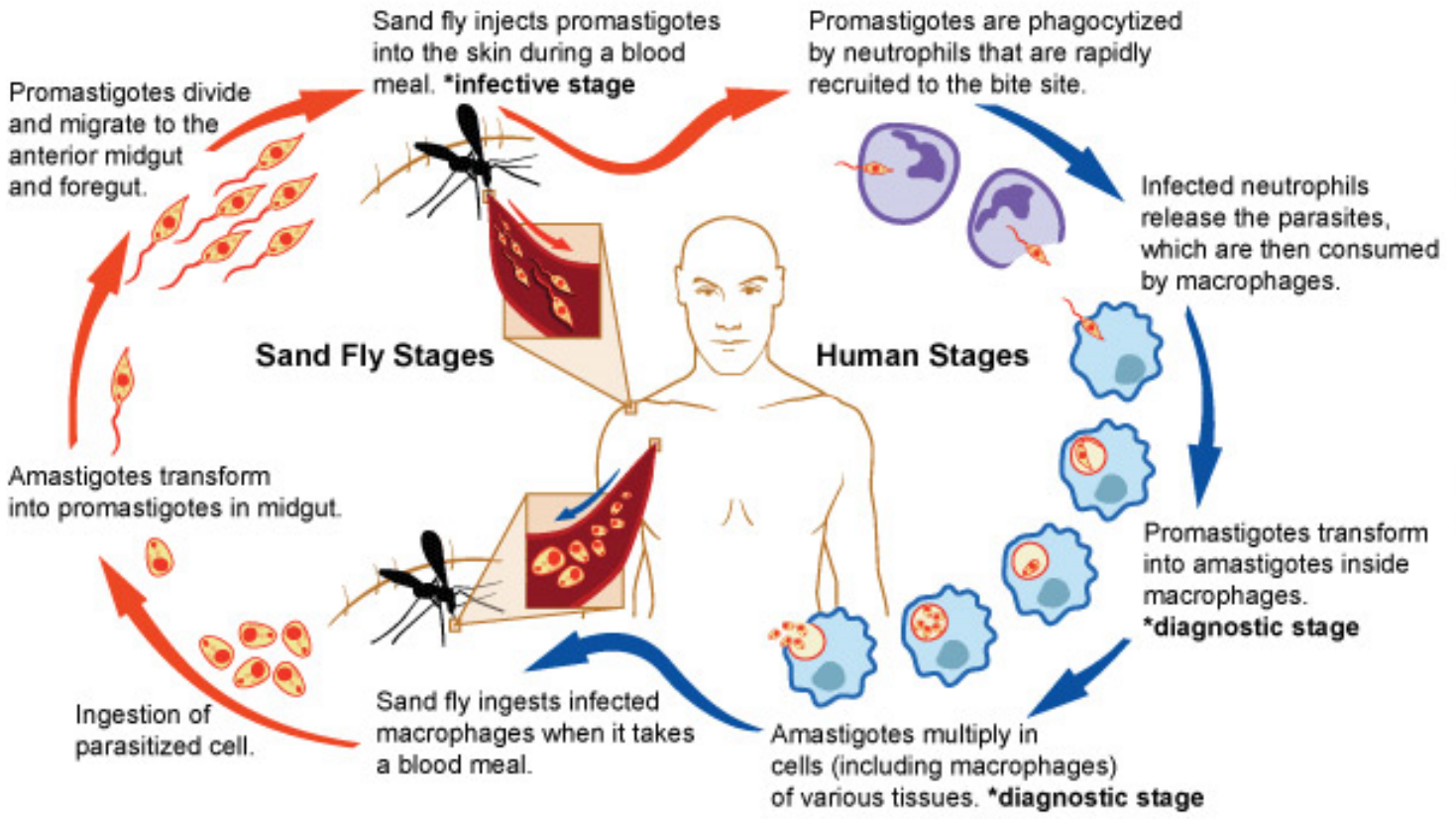


Figure 1.5 Life cycle of the *Leishmania* parasite

Source: National Institute of Allergy and Infectious Disease

relationship between mammal and invertebrate development implies that leishmaniasis was established at 50 Mya. The worldwide dissemination of *Leishmania* likely followed vectors and hosts together as sand flies cannot migrate large distances. The genus most likely originated in South America 46 -36 Mya from where it migrated to Asia and then on to Africa, India and Europe as indicated by DNA sequencing showing a divergence of the visceral strains *L.donovani* and *L.infantum* at ~1 Mya, with further divergence of infraspecific genetic groups between 0.4 and 0.8 Mya (Lukes *et al*., 2007). The *L. donovani* complex possibly migrated both ways between East Africa and Asia probably via immigration or slaves. *L.infantum*, completed the circle 500 years ago through migration from Europe, back to South America by European settlers (Singh and Roy, 2009). *L.chagasi* is now synonymous with *L.infantum.*

Although there are historical records of CL dating back to 650 BC, when old world CL was described on tablets of King Ashurbanipal of Assyria, there were no descriptions of VL until 1824 AD from Jessore, Bengal. The disease, which was named kala azar – Hindi for 'black disease/fever', spread rapidly by road and rail and had a devastatingly high mortality of 30 % within affected populations (Singh and Roy, 2009). It was initially presumed to be a form of quinine-resistant malaria. The identification of the causative parasite did not occur until almost simultaneously discovered in the spleen of patients who been resident in West Bengal, by William Leishman and Charles Donovan. It was correctly identified as being closely related to the trypanosomes and the given the name *Leishmania donovani* (Ross, 1903).

1.1.3 Leishmania biology and life cycle

The life cycle of the *Leishmania* species is depicted in Figure 1.5. The parasite is transmitted, in promastigote form through the bite of an infected sand fly and ingested at

the site by rapidly recruited neutrophils. Infected neutrophils undergo apoptosis and release the parasites prior to ingestion by the macrophage. The promastigote differentiates into amastigote form in the phagolysosome of the macrophage and the amastigote multiplies by binary fission. Further macrophages are infected directly by amastigotes released from lysis of heavily infected macrophages (Murray *et al*., 2005). When a sand fly takes a blood meal from an infected host, infected macrophages are taken up and lysed in the sand fly gut. Here, the amastigotes differentiate into promastigotes which further differentiate in the chitinous foregut into non proliferative metacyclic forms and migrate to the proboscis. Infected sand flies are thought to have a 'blocked' gut which promotes them to probe the skin more frequently and more persistently particularly when metacyclic forms are present, thus enhancing transmission of the highly motile metacyclic forms to the mammalian host (Rogers and Bates, 2007). Approximately 1000 promastigotes are transmitted in a bite within a proteophosphoglycan (PPG) plug which promotes pathogenicity and increased parasite burden within the host (Rogers *et al*., 2004).

Table 1.1 summarises the hosts *Leishmania* inhabit which act as reservoirs for transmission from the sand fly to humans in these regions. The *Leishmania* parasite can also be vertically transmitted from mother to child and parenterally through blood transfusion, needle sharing and laboratory accidents (Herwaldt, 1999). These are uncommon modes of transmission.

1.1.3.1 Leishmania genome

The first full *Leishmania* genome to be assembled was that of the CL-causing species *Leishmania major* in 2005 (Ivens *et al*., 2005). It is a 32.8 megabase haploid genome with 36 chromosomes. Later comparison between *L.major* and *L.infantum* genomes indicated that gene deletion and pseudogene formation accounted for the main differences. These

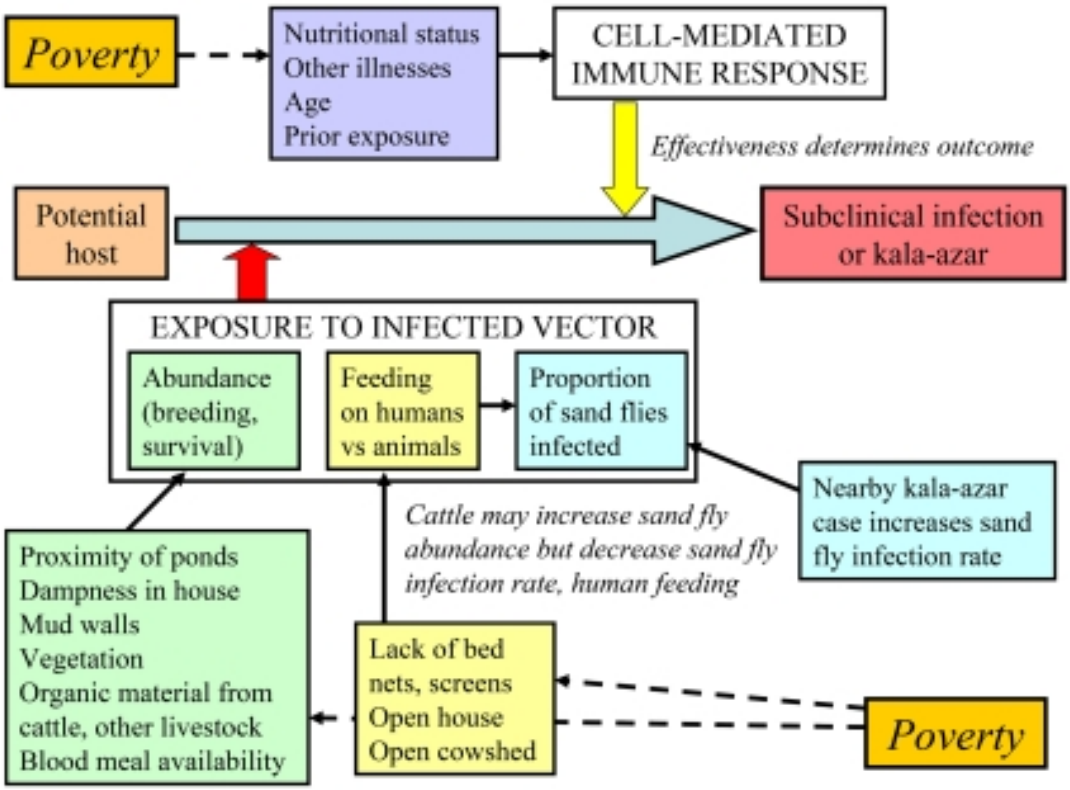


Figure 1.6 Factors influencing infection and clinical course in visceral leishmaniasis

Proximity to cattle and other VL (kala-azar) cases increases risk of infection which can be mediated by use of bednets and good quality housing. Cell mediated immunity is thought to be the most influential in progression to clinical VL. Figure from (Bern *et al.*, 2010)

included key genes involved in host pathogen interactions and intra-macrophage survival (Peacock *et al*., 2007). The availability of the genome has been, and will continue to be valuable, for many insights into biology and pathogenesis (Stuart *et al*., 2008).

1.1.4 Visceral leishmaniasis clinical features

The clinical features of *Leishmania* infection are related to both parasite and host factors. Only 1 in every 5-10 infections with *L.donovani and L.infantum* develop into clinical VL disease. The reasons behind development of clinical infection are not fully understood but risk factors identified include age, ethnic origin, sand fly exposure levels, proximity to a previous case, poverty, housing conditions, low red meat intake and low zinc and retinol levels (Bern *et al*., 2010; Bern *et al*., 2007; Bucheton *et al*., 2002) (Figure 1.6). The observation of familial clustering and high sibling relativity point towards genetic factors behind the development of infection. In Sudan, strong correlations have been identified between SLC11A1 (formerly NRAMP1 (natural resistance associated macrophage protein)) and susceptibility (Mohamed *et al*., 2004). There was no correlation between this protein and susceptibility found in recent work from the *Leishmania* genetics consortium in India. It identified a strong correlation with polymorphisms in the HLA-DRB1-HLA-DQA1 HLA class II region (Fakiola *et al*., 2013). Innate immunity and T cell responsiveness is also thought to be integral to response to infection (Murray *et al*., 2005).

In clinical visceral leishmaniasis the incubation period is weeks to months following which patients can follow an acute or chronic course with classic symptoms of fever, malaise, anorexia and eventual cachexia. In India this is accompanied in a proportion of cases with skin darkening which gave the condition its name of “kala azar” which means “black fever” in Hindi. Patients display signs of hepatosplenomegaly due to a combination of inflammation and a heavy burden of parasitised macrophages. In Africa this is

accompanied by pronounced lymph node enlargement, a clinical sign almost absent in Indian VL. Laboratory markers are of pancytopenia (anaemia, thrombocytopenia and leucopenia with a relative lymphocytosis and monocytosis) a hypergammaglobulinaemia and hypoalbuminaemia. Death is usually from bacterial co-infection due to gradual disabling of the immune system with progressive infection (Herwaldt, 1999).

Relapse of the signs and symptoms of VL can occur, most commonly in the 6 months following treatment, but in some cases more than 10 years post initial infection (unpublished data and personal communication Shyam Sundar). There is a debate whether these late relapses are reinfection or recrudescence but parasites can persist intra-cellularly post successful treatment and a VL infection should confer lifelong immunity so reinfection is less likely (Murray *et al*., 2005).

A proportion of treated cases, 50% in Sudan (Zijlstra *et al*., 1995) and 10-15% in India (Desjeux *et al*., 2013), go on to develop PKDL (Figure 1.1, Panel D), characterised by highly parasitized skin lesions on sun exposed areas. This happens within months in Africa but on average not for 1-2 years in India. Although the lesions are non-painful macular, papular or nodular lesions that can spontaneously regress, treatment is advised as PKDL is an important reservoir for VL transmission.

1.1.5 *Leishmania* immunology

The interplay between *Leishmania* and the host immune system is essential for parasite survival. The host defences mounted against the leishmania parasite are responsible for many of the clinical symptoms and the manipulations of the immune system by the parasite are responsible for the immunosuppression and susceptibility to co-infections (Murray *et al*., 2005). Metacyclic promastigotes evade complement(C)-mediated lysis by changing

their cell surface membrane's lipophosphoglycan (LPG) which prevents the insertion of the membrane attack complex (MAC) together with the enhanced synthesis of gp63 which inactivates C3b to iC3b. The presence of iC3b stimulates phagocytosis by macrophages – the leishmania cell of choice (Späth *et al*., 2003).

Within the macrophage the phagosome fusion with the lysosome is transiently delayed by the surface LPG to allow for differentiation from promastigote to amastigote. The resulting parasitophorus vacuole has an acidic pH of 5.5 and hydrolytic activity which are thought to be survived by the parasite through further cell surface changes (Sacks and Sher, 2002). In an immunocompetent host the triggering of the T cell helper 1 (Th) system is responsible for activating the macrophage into a cytocidal state to produce reactive nitrogen and oxygen species. However, the intra- macrophage amastigote arrests the JAK/STAT signal pathway by action on SHP1 thus actively inhibiting the production of interleukin-12 (IL-12) which stimulates the production of interferon–γ (IFN–γ) and the Th1 response (McDowell and Sacks, 1999). In addition to affecting dendritic cell function, which is vital for antigen presentation, *Leishmania* encourage the immunosuppressive Th2 response, including IL-4, IL-10, IL-13 and Transforming growth factor-β, which dampens the Th1 response and deactivates macrophages. Although a balance favouring a Th2 response is strongly evident in experimental infections the relationship is less clear cut in human VL (Murray *et al*., 2005). However, even in the presence of T cell dependent immune responses producing self-healing or asymptomatic infection, intracellular infection is thought to be life-long as residual parasites persist in macrophages. The importance of a T-cell response has been well documented experimentally (Sacks and Anderson, 2004) and is clinically demonstrated by the poor prognosis of VL patients co-infected with human immunodeficiency virus (HIV) which targets CD4+ T cells (Cota *et al*., 2013).

1.1.6 Leishmania diagnosis and screening

The gold standard for *Leishmania* diagnosis is demonstration of Leishman-Donovan (LD) bodies following microscopic examination of Giemsa-stained splenic aspirates. This method has an excellent specificity and sensitivity ranges from 93.1-98.7%. Unfortunately, it is invasive and technically challenging and without appropriate precautions can carry the risk of fatal haemorrhage (Sundar and Rai, 2002). Bone marrow biopsy can also diagnose VL through visualisation of LD bodies but its sensitivity is low: between 52 -85% (Srivastava *et al*., 2011a).

A serological test, rK39, against a recombinant antigen of a 39 amino acid repeat in *L.chagasi* was initially shown to be effective for diagnosis in an enzyme linked immunosorbent assay (ELISA). Since then many different commercial dipsticks have become available. A meta- analysis of sensitivity and specificity in South Asia is 97.1% and 95.3%, respectively (Chappuis *et al*., 2006). However, for unknown reasons, in East Africa, the sensitivity and specificity of rK39 are considerably lower at 79.0% and 85.2%, respectively. The rK39 is the recommended test for diagnosis on the Indian subcontinent.

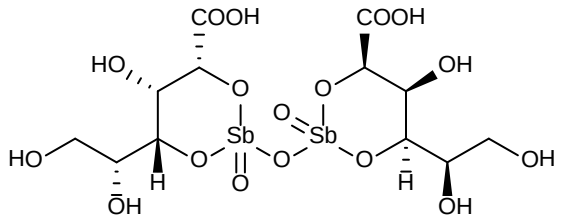
The direct agglutination test is a semi quantitative test where dilutions of the patient's blood or serum are mixed with fixed, stained promastigotes in a v-shaped well micro plate. Agglutination is visible to the naked eye if antibodies to the parasite are present. This test also has a higher sensitivity and specificity in South Asia versus East Africa but its performance is better than the rK39 in Africa with sensitivity and specificity of 93.2% and 96.1% respectively. The direct agglutination test is in routine use in Sudan (Chappuis *et al*., 2006).

Other techniques such as ELISA, immunoblotting and immunofluorescence antibody test (IFAT) are expensive and time consuming and not for routine use in the field. The use of urinary antigens as a minimally invasive form of diagnosis shows promise. The use of PCR is a growing field in *Leishmania* diagnostics and field work and has been particularly useful in species identification. The techniques mainly use primers which target multicopy genes such as rRNA and kinteoplastid DNA, however as yet no techniques are in routine use in the field for VL (Srivastava *et al*., 2011a).

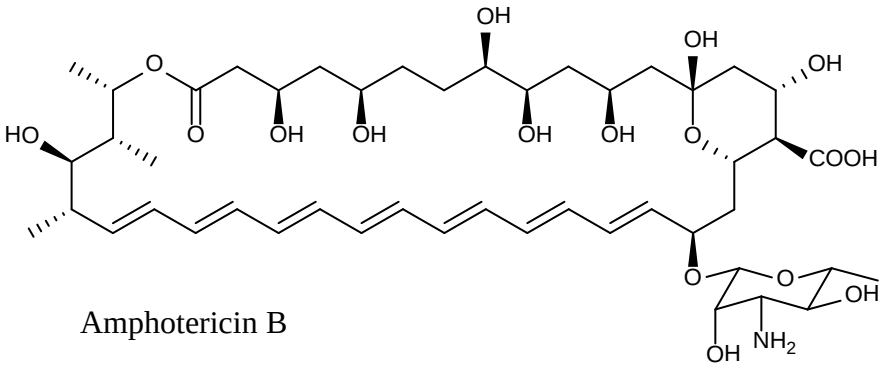
The prevalence of sub-clinical infection within endemic communities presents a diagnostic challenge. Studies of most diagnostic tests show higher specificities when the control group are healthy rather than a controls living in an area endemic for VL (Chappuis *et al*., 2006). Interestingly though, a longitudinal study in Bihar and Nepal showed that seropositivity and/or rK39 at high titres was associated with an increased risk of developing clinical VL (Hasker *et al*., 2014) and that seroconversion to negative serology does occur despite the fact that infection with VL is thought to be permanent (Hasker *et al*., 2013).

The toxicity of anti-leishmanial treatments (Section 1.1.7) means that accurate diagnosis is essential. The Foundation for Innovative Diagnostics (FIND) brings together professionals from all fields to try and accelerate the development of new diagnostics. It is currently leading work evaluating the PCR technique Loop mediated isothermal amplification (LAMP) and is looking at ELISA assays to establish a serological test as a test of cure at the end of treatment[4].

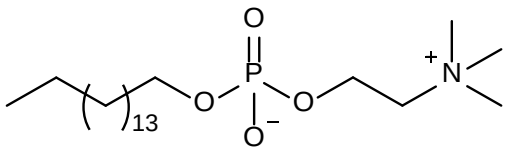
[4] http://www.finddiagnostics.org/programs/hat-ond/leishmaniasis/



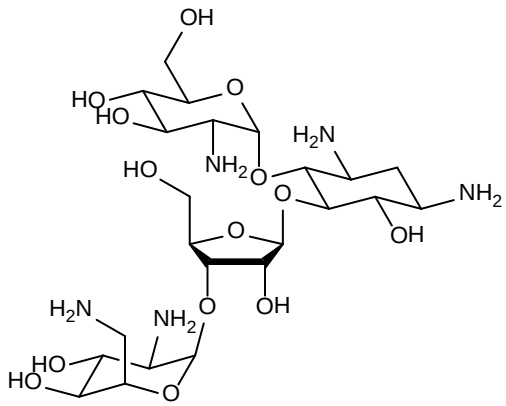
Sodium stibogluconate (Pentostam)



Amphotericin B



Miltefosine



Paromomycin

Figure 1.7 Chemical structures of current anti-leishmanial therapies

Table 1.2 Current visceral leishmaniasis treatments

Adapted from: (Guerin *et al.*, 2002; Griensven *et al.*, 2010; Balasegaram *et al.*, 2012)

Drug	Delivery route	Dose	Course length	Reported efficacy	Cost (US$)	Toxicity	Other issues	Combination with:
SSG, MA (pentavalent antimonial)	IM	20 mg kg^{-1} d^{-1}	20-30 d	35 -95% (geographic variation)	53 – 198	3-5% mortality Morbidity cardiotoxicity, nephrotoxicity, hepatic and pancreatic toxicity	Poor efficacy in HIV. Drug quality. Painful injections.	Paromomycin
Amphotericin B (polyene antibiotic)	Slow IV infusion	Total 15 mg kg^{-1} (1 mg kg^{-1} alternate days)	30 d	>97% all regions	280- 480	Renal failure Cardotoxicity	Monitoring of toxicity	
Liposomal Amphotericin B (polyene antibiotic)	Slow IV infusion	10 -30 mg kg^{-1}	1- 10 d	>97% India single dose, 91 % Africa	126 -378 (WHO negotiated)	Minimal	Price, heat stability, slow infusion	Miltefosine
Miltefosine (alkylphospho-lipid)	Oral	2-2.5 mg kg^{-1} d^{-1}	28 d	94% India, other regions not fully establised	65 -150 (WHO negotiated)	Teratogenicity Nausea and vomiting common	Long half life. Poor compliance	L-AMB
Paromomycin (aminoglycoside)	IM	15 mg kg^{-1} d^{-1}	21 d	>90% efficacy in South Asia	$15	Nephrotoxicity Ototoxicity Hepatotoxicity	Painful injections Variable efficacy	SSG

IM intramuscular IV intravenous SSG Sodium stibogluconate MA Meglumine antimonate

1.1.7 VL treatment overview

The chemotherapeutic arsenal against VL is currently limited to four main drugs: pentavalent antimonials, amphotericin B compounds, miltefosine and paromomycin (Croft, 2008; den Boer *et al*., 2009)(Table 1.2) and (Figure 1.7). There are significant issues with each drug for use in low income countries including efficacy, toxicity, cost and storage. Antimonial based compounds are the main subject of this study and will be discussed in detail in Section 1.2.

1.1.7.1 Amphotericin

Amphotericin B was originally isolated from *Streptomyces nodosus* and has been used since the mid-20th century against fungal infections. Its mechanism of action is through binding to membranous ergosterol, thus forming membranous pores, which allows for loss of vital intracellular constituents. This mechanism unfortunately also leads to marked renal and ototoxicity (Ng *et al*., 2003). In the 1980s researchers were able to capitalise on the discovery of liposomes (New *et al*., 1981) for drug delivery and liposomal amphotericin compounds (L-AMB) were developed with a more favourable toxicity profile. Amphotericin B deoxycholate was first found to be effective against VL in 1963 but was only shown to be an effective first line treatment in South Asia in the 1990s (Mishra *et al*., 1994). Subsequently, differing regimes with L-AMB have shown excellent results in Asia with initial cure rates exceeding 95% (Burza *et al*., 2014a; Sundar *et al*., 2010). Although L-AMB has a lower efficacy in the VL endemic African countries (91% initial cure rate) (Salih *et al*., 2014), it has an important place in treating vulnerable groups e.g. in pregnancy or HIV co-infection (Balasegaram *et al*., 2012).

The 6-month cure rate of 96% for single dose liposomal amphotericin (Sundar *et al*., 2010) is particularly attractive for practical and compliance reasons and negotiations are

underway for this to be the primary drug in the VL elimination programme (Section 1.1.8.5). The main fear of this single dose regime is resistance development. However, although amphotericin resistant parasite strains have been easily selected *in vitro* in promastigotes, (Mbongo *et al*., 1998) only one case report of amphotericin resistance in a clinical isolate (Srivastava *et al*., 2011b) has been published. Reassuringly, assessment of sensitivity to AMB in successive clinical isolates from an HIV co-infected patient following repeated AMB administration showed no change in drug sensitivity (Durand *et al*., 1998). The efficacy of L-AMB in Africa is lower and it is not recommended as a first line treatment (World Health Organization, 2010).

The cost of L-AMB, as well the need to keep it in a cold chain, is an impediment to its widespread use. However, through preferential pricing organised by the WHO, there has recently been a promise from Gilead to provide L-AMB at $18 per vial – more than ten times less than the cost of private purchase (World Health Organization, 2010).

1.1.7.2 Miltefosine

Although the establishment in 2002 of the alkylphospholipid miltefosine as the first oral drug for VL was greeted with great excitement (Sundar *et al*., 2002), a decade later its cure rate at 6 months had fallen from 94% to 90% (Sundar *et al*., 2012) with 10% more late relapses (>6 months) also reported (Rai *et al*., 2013a). Miltefosine, originally developed as an anti-cancer drug, is thought to inhibit vital cell signalling pathways and is metabolised by the phospholipid pathways (Dorlo *et al*., 2012). It has a half-life of approximately 7 d, giving it high resistance potential. Interestingly though, minimal correlation has been seen between miltefosine treatment failure and parasite susceptibility in current *in vitro* assays (Rai *et al*., 2013a) leading to speculation about the mechanism of treatment failure. In a recent trial, greater than half of patients reported significant gastro-intestinal side effects

which is a major issue for compliance in unsupervised treatment regimens (Sundar *et al*., 2012). Its teratogenicity is also a major issue. Although initially part of the VL elimination programme, in the WHO 2010 report (World Health Organization, 2010), these above developments meant that miltefosine was not recommended as a first line treatment in any region.

1.1.7.3 Paromomycin

Paromomycin is an aminoglycoside antibiotic which inhibits protein synthesis by directly binding to ribosomal RNA (Fernandez *et al*., 2011). It is effective against VL and CL as well as having toxicity against other protozoans: amoeba and cryptosporidia. It is very attractively priced and an efficacy of 94% as monotherapy and non-inferiority to AMB has been demonstrated in India (Sundar *et al*., 2007). However, paromomycin is only recommended for use in combination therapy due to fears of resistance development (Hendrickx *et al*., 2012) and poor efficacy as a monotherapy in East Africa (<85% cure)(World Health Organization, 2010).

1.1.7.4 Combination therapy

Different combinations of the above drugs have been trialled in both India and Africa with a view to shortening lengthy treatment regimens (Musa *et al*., 2012; van Griensven J. *et al*., 2010). Experience from other infectious diseases including HIV and tuberculosis (TB) has shown that combination therapy can help to prevent resistance (Gazzard, 2001). However a cautionary note is that resistance to all combinations has been easily selected for over a 10 week period in the laboratory (Garcia-Hernandez *et al*., 2012). Due to the excellent efficacy of L-AMB, combination regimes are not being routinely used in India, whereas in Africa SSG and PMM is the first line recommended treatment of lasting just 17 days

(World Health Organization, 2010). Further work is required to identify the most appropriate, effective and safe regimes.

There has been some interest over the years in combining conventional treatment with immunological adjuncts due to VL's profound effect on the immune system (Section 1.1.5) and additionally due to the immunological arm of the mode of action of antimonials (Section 1.2.4). In a small trial in Bihar comparing IFN-γ with SSG and SSG alone there was an accelerated rate of parasite clearance in the IFN-γ treated group (Sundar *et al*., 1995). Further trials in Bihar of this combination were hindered due to low response in both arms likely due to high levels of antimonial resistance (Sundar *et al*., 1997). Experimental evidence showing the inhibitory effect on parasite growth of the monoclonal antibody anti IL-10 on fresh splenic aspirates (Gautam *et al*., 2011) and the dose sparing effect of anti IL-10 combined with pentavalent antimony in murine models (Murray, 2005) provides support for anti IL-10 as a therapeutic target in human VL.

1.1.7.5 Outcome monitoring

As mentioned in Section 1.1.2 there is under reporting of the incidence of VL. As well as improving incidence figures, record keeping is vital for control and resistance monitoring. If treatment outcomes are accurately recorded at all levels from district health care centres to tertiary hospitals it can help to preserve essential drug function. VL care can learn from TB care in this and a recent pilot study using a retrospective quarterly cohort monitoring model in Nepal has shown good feasibility (Ostyn *et al*., 2013). This design will catch the late relapses which have been occurring with the use of Miltefosine and L-AMB (Burza *et al*., 2014b).

1.1.7.6 Drug development

Better treatment options for VL are desperately needed. The Drugs for Neglected Diseases Initiative which is a collaborative, not for profit research and development organisation have set out an ideal product profile for new therapeutics. Products should be a once daily oral formulation that is active against all species and resistant strains, compatible for combination therapy, < $10/course, safe in pregnancy and immunocompromised persons and stable in tropical conditions[5].

Amphotericin B, miltefosine, and paromomycin were all initially developed for other conditions. Repurposing of drugs could continue to provide exciting new VL therapies as 2 of the most promising candidates fexinidazole (clinical trial phase II/II HAT) and PA824 (clinical phase II TB) have recently been shown to have excellent efficacy *in vitro* and *in vivo* against *L donovani* (Wyllie *et al*., 2012) (Patterson *et al*., 2013). Due to preliminary work on fexinidazole for HAT, fexinidazole has been able to enter directly into phase II clinical trials against VL in Sudan[6]. VL-2098 is another promising compound[7] .

1.1.8 Sand flies and VL control

There are many factors that a country requires for adequate control of vector borne diseases: peace, long-term political commitment, finance, robust control methods and public health education. The control methods must address all aspects of the *Leishmania* life cycle including the parasite, the human host, any animal reservoirs if applicable and the sand fly vector (Chappuis *et al*., 2007). Accurate diagnosis and effective treatment (Section 1.1.7 and 1.1.7) are essential but without focus on the sand fly vector VL control will not be achieved.

[5] http://www.dndi.org/diseases-projects/diseases/vl/tpp/tpp-vl.html
[6] http://clinicaltrials.gov/show/NCT01980199, accessed 11.04.14
[7] http://www.dndi.org/diseases-projects/portfolio/vl-2098.html

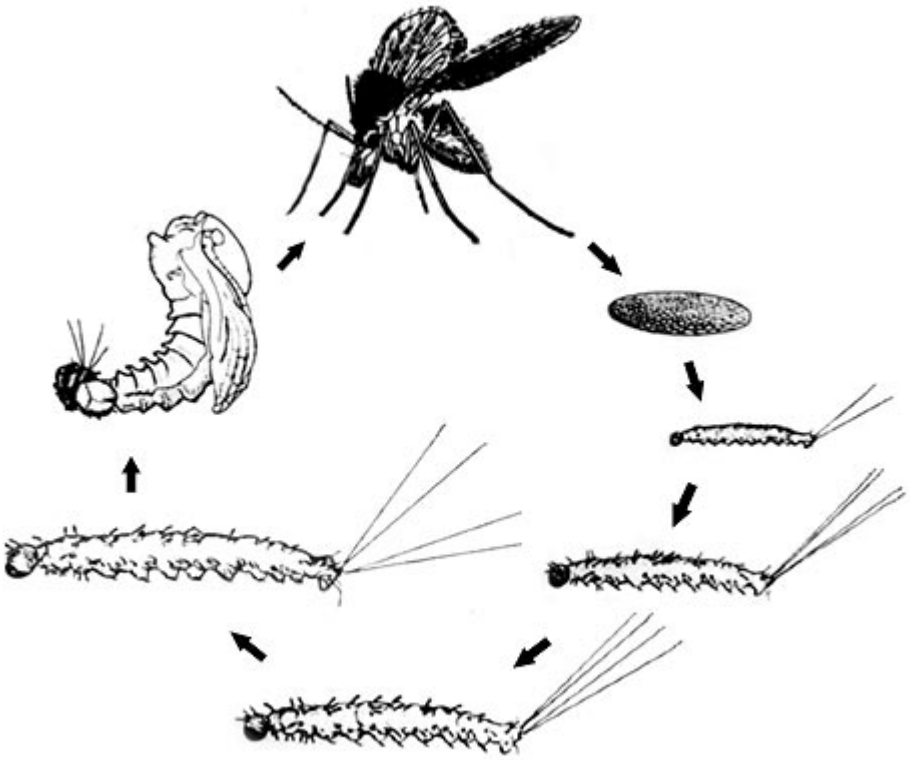


Figure 1.8 Life cycle of a sand fly

The life cycle occurs in a terrestrial environment with eggs being laid in moist organic matter. There are 4 larval instars prior to pupation and adult emergence. Source: pcwww.liv.ac.uk/leishmania/life_cycle_habitats.htm

1.1.8.1 Sand fly life cycle, feeding and breeding

Ninety three out of 800 species of sand fly are proven or possible vectors of *Leishmania* (World Health Organization, 2010). The main species transmitting human pathogenic *Leishmania* are summarised in Table 1.1. The lifecycle of a sand fly is shown in Figure 1.8. The eggs develop in moist organic matter and this development is hastened by high temperatures. The larval stage is 3 weeks, their food source during this time is largely unknown though rat faeces has been implicated (Mascari *et al*., 2013). Adult flies emerge after pupation of 10 days.

Only female sand flies take blood meals and are therefore involved in transmission, requiring the blood for egg development. Both male and female sand flies require sugar meals and many species feed on aphid honey dew as a sugar source. During the day sand flies rest in cool, humid niches including cracks in poorly maintained houses, bedrooms, latrines and dense vegetation. Females bite at night time but vary between species on whether they bite in/outdoors (endo/exophagic) and rest to mature their eggs in/outdoors (endo/exophilic) (Lane, 1993). These distinctions are important for vector control measures such as indoor residual spraying and bed net usage. Sand flies cannot fly at speeds greater than 1 m s^{-1}. They can move 1 km in a night but do not tend to disperse greater than 1 km in a lifetime. The sand fly life expectancy is mainly unknown but is thought to be at least 1.5 ovarian cycles (approximately 9 days) (Dye *et al*., 1987). Factors which favour sand fly breeding in the Indian subcontinent are higher indoor temperature and relative humidity marshy land and orchard/settlement and areas with higher soil pH and moisture content (Kesari *et al*., 2011; Lane, 1993).

1.1.8.2 Vector control methods

Vector control mechanisms must be designed on the known feeding and resting habits of the sand fly species being targeted. In the 1950s and 1960s VL was almost eliminated in India as a secondary result of the National Malaria Eradication programme which employed the technique of indoor residual spraying (IRS) with the organochloride insecticide dichlorodiphenyltrichloroethane (DDT) (Ostyn *et al*., 2008). A recent review of vector control in the Indian subcontinent concluded that IRS can dramatically reduce sand fly density (Picado *et al*., 2012). A recent Indian trial of long lasting insecticide (deltamethrin) treated bed nets (LN) did not show an impact on VL transmission, calling into question the presumed endophilic and endophagic habits of *P.argentipes* (Picado *et al*., 2010).

Manipulating the food source of sand flies to include a form of paratransgenetic *Bacillus subtilis,* which produces leishmanicidal molecules, is one of the novel mechanisms of control that are being developed (Hurwitz *et al*., 2011). Further study on sand fly behaviour and prospective studies on VL control is required.

1.1.8.3 Reservoir Control

In areas where the life cycle involves mammals various methods have been employed. The widespread culling of infected dogs is now controversial and treatment has not been shown to be infective and carries the risk of generating resistant parasites. Deltamethrin impregnated dog collars are effective (Chappuis *et al*., 2007). In areas where the life cycle is anthropogenic, PKDL is an important reservoir as the lesions are highly parasitised. Active case finding is important for PKDL and is particularly effective for VL in areas with little public health intervention (Mondal *et al*., 2009). The impact of sub-clinically infected persons on transmission is a controversial topic (Singh *et al*., 2014).

1.1.8.4 Vaccination

There is no available vaccine for VL but the strong immunity that follows cured infection implies that vaccination is highly possible. Vaccination should be attractive economically to affected countries, not only due to the high burden of morbidity and mortality that VL carries, but also because for most families the cost of treatment and loss of work time substantially increases their poverty index (Chappuis *et al*., 2007). However, vaccination has not been taken up by any industrial company (Kaye and Aebischer, 2011).

The century old practice of 'Leishmanization' where naïve patients were deliberately infected with *L. major*, although providing further hope for the vaccine approach, does not confer protection for VL. There are 4 developed vaccines currently in clinical development. The most developed, Leish-111f, a fusion protein of the thiol-specific antioxidant protein tryparedoxin peroxidase, stress inducible protein 1 and elongation initiation factor, has entered phase II clinical trials as a therapeutic vaccine. Ten other vaccination candidate proteins are undergoing pre-clinical research (Alvar *et al*., 2013). There is hope that this post genomic, bio-informatic era will bring new promising vaccine candidate molecules (Kaye and Aebischer, 2011).

1.1.8.5 Visceral Leishmaniasis Elimination Initiative

The countries of India, Nepal and Bangladesh joined together in 2005 to form the Visceral Leishmaniasis Elimination Initiative (Sundar *et al*., 2008). At this point elimination by 2015 was thought possible because in India, humans are the only disease reservoir, the sand fly is responsive to IRS, the rK39 is an effective diagnostic and miltefosine, the first oral drug for VL, had recently been registered. However, although the rK39 is working well, there are issues with miltefosine (1.1.6) and IRS programmes have been sub optimal.

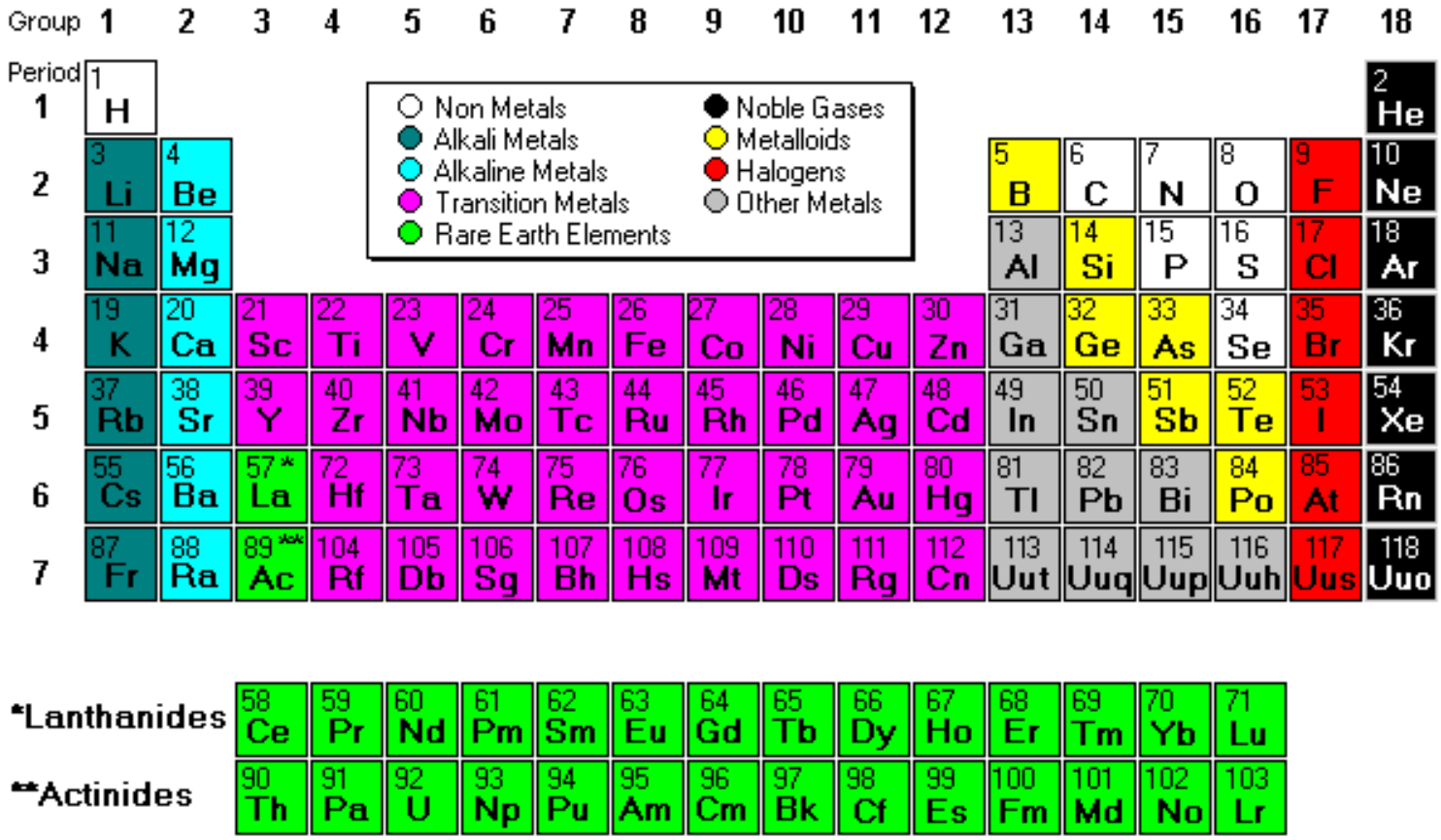


Figure 1.9 Periodic table

Yellow squares are metalloid elements. Source: modelscience.com/PeriodicTable.html

Table 1.3 Properties of the elements arsenic and antimony

Property	**Antimony**	**Arsenic**
Symbol	Sb	As
Electronic configuration	[Kr] 4d10 5s2 5p3	[Ar] 4s2 3d10 4p3
Atomic number	51	33
Atomic mass	121.76	74.92
Melting point	630.8 ^{0}C	816.8 ^{0}C
Boiling point	1587 ^{0}C	613 ^{0}C
Form found in nature	Stibnite (antimonite), valentinite	Arsenic, orpiment, realgar, arsenopyrite, scorodite
Relative abundance	0.2 ppm	1.8 ppm

There is hope with single dose L-AMB but there is still a long way to go for elimination to be achieved (Matlashewski *et al*., 2011).

1.2 Antimony

1.2.1 Description

Antimony is a chemical element with the symbol Sb and atomic number 51, belonging to the metalloids and Group 15 of the Periodic Table of Elements (Figure 1.9) (Table 1.3). Metalloids are chemical elements with properties in between metals and non-metals (Sekhon, 2013). They are too brittle to be structural and are only weak conductors of electricity. As a group, the metalloid's main uses are in alloys, biological agents, retardants, glasses, optical storage, pyrotechnics, semiconductors and electronics.

Antimony mainly occurs in nature as stibnite/antimonite (Sb_2S_3) or valentinite (Sb_2O_3) found commonly in copper, silver and lead ores, mined from Bolivia, China and Africa (McCallum, 1977). It is a silvery lustrous grey metal, stable at room temperature but reacts with oxygen if heated. It can occur in the oxidation states of –III, 0, III and V but is mainly found in III and V (Filella *et al*., 2002). Its relative abundance is 0.2 parts per million (ppm) where silver is 0.007 ppm and arsenic is 1.5 ppm. Antimony is mined mainly for use as an alloy, a semi-conductor and as a flame retarding additive
(Sundar and Chakravarty, 2010).

1.2.2 History of therapeutic use

Antimony has a long history of use as a medicine. The ancient Egyptians used it for fevers, skin irritations and internal tumours as well as for cosmetic purposes. Famously the reaction between wine and antimony goblets meant it was used as an emetic to allow for

over eating in banquets in the Roman times. The compound potassium antimony tartrate was not officially discovered until the early 1600s by Adrian von Mynischt the German biochemist. Antimony was popular throughout the 16th to 17th century as the 'universal medicine' for pneumonia, melancholy, chest pains, plague and cancers (Yan *et al*., 2005). It was paraded by a Benedictine monk in the publication 'The Triumphal Chariot of Antimony' (Valentinus and Kerkringius, 1893) written under the pseudonym Basil Valentine which extensively lists its medicinal and toxic properties. Antimony became less popular in the 18th and 19th century due to its toxicity but returned to favour in the 20th century when its anti-parasitic effects against *Leishmania* and *Schistosoma* were discovered (Sekhon, 2013).

When Leishman and Donovan first described the *Leishmania* parasite at the beginning of the twentieth century there was not any known effective treatment. The sensitivity of the related parasite trypanosomes to the arsenic compound Atoxyl, discovered by H.W.Thomas (Thomas, 1905) and subsequently confirmed and developed by Paul Ehrlich (Frith, 2013), prompted the young Brazilian physician, Gaspar Vianna, to use potassium antimony tartrate against MCL (Goodwin, 1995). Independently, Leonard Rogers started using the trivalent compound against visceral leishmaniasis and in 1921, 20,000 injections were administered in Assam with an 88% cure rate. However the toxicity of severe vomiting was such that over a third of patients did not complete their treatment course and distrust of the medical profession developed (Singh and Roy, 2009).

The issue of toxicity was improved in India by Bramachari's development of the pentavalent urea stibamine (Brahmachari, 1989). This compound had a cure rate of 90% and significantly less toxicity (Singh and Roy, 2009). By 1933, 328,591 patients had been cured. Later in the twentieth century urea stibamine was replaced by sodium

stibogluconate developed by Len Goodwin at the Wellcome laboratories, Euston road, by monitoring response in leishmania parasite load in freshly harvested hamster spleen (Goodwin, 1995).

Pentavalent antimonials, in the form of sodium stibogluconate (generic SSG, Albert David or Pentostam, GlaxoSmithKline) or meglumine antimonite (Glucantime™, Sanofi) are currently used worldwide in the treatment of both VL and CL (World Health Organization, 2010). Up until the late 1990s there were issues with different batches of generic SSG having unexpectedly high toxicities, likely due to the presence of contaminating trivalent antimony which is reflected by increased osmolarity (Sundar *et al*., 1998). Since then the International Dispensary Association (IDA) have taken on regular quality testing. Generic SSG has been shown in clinical trial format to be non-inferior to the significantly more expensive GSK compound Pentostam (Veeken *et al*., 2000) and this is the form used in most developing countries.

Although trivalent antimonial preparations are active against schistosomiasis, they have seldom been used since the 1970s as the discovery of Praziquantel provided a more effective and less toxic alternative (Sundar and Chakravarty, 2010).

1.2.3 Antimony in the environment and toxicities

The general population in the US are exposed to low levels of antimony at 5 μg day^{-1} from both inhalation and consumption of food and water. Antimony is present in aerobic fresh water in the pentavalent state and in anaerobic conditions in trivalent state, usually at a level of 0.1 to 0.2 μg L^{-1}. Less than 5 μg L^{-1} is the WHO recommendation[8]. Toxicity therefore usually stems from exposure via occupation (workers in the antimony production

[8] http://www.who.int/water_sanitation_health/dwq/chemicals/antimony.pdf

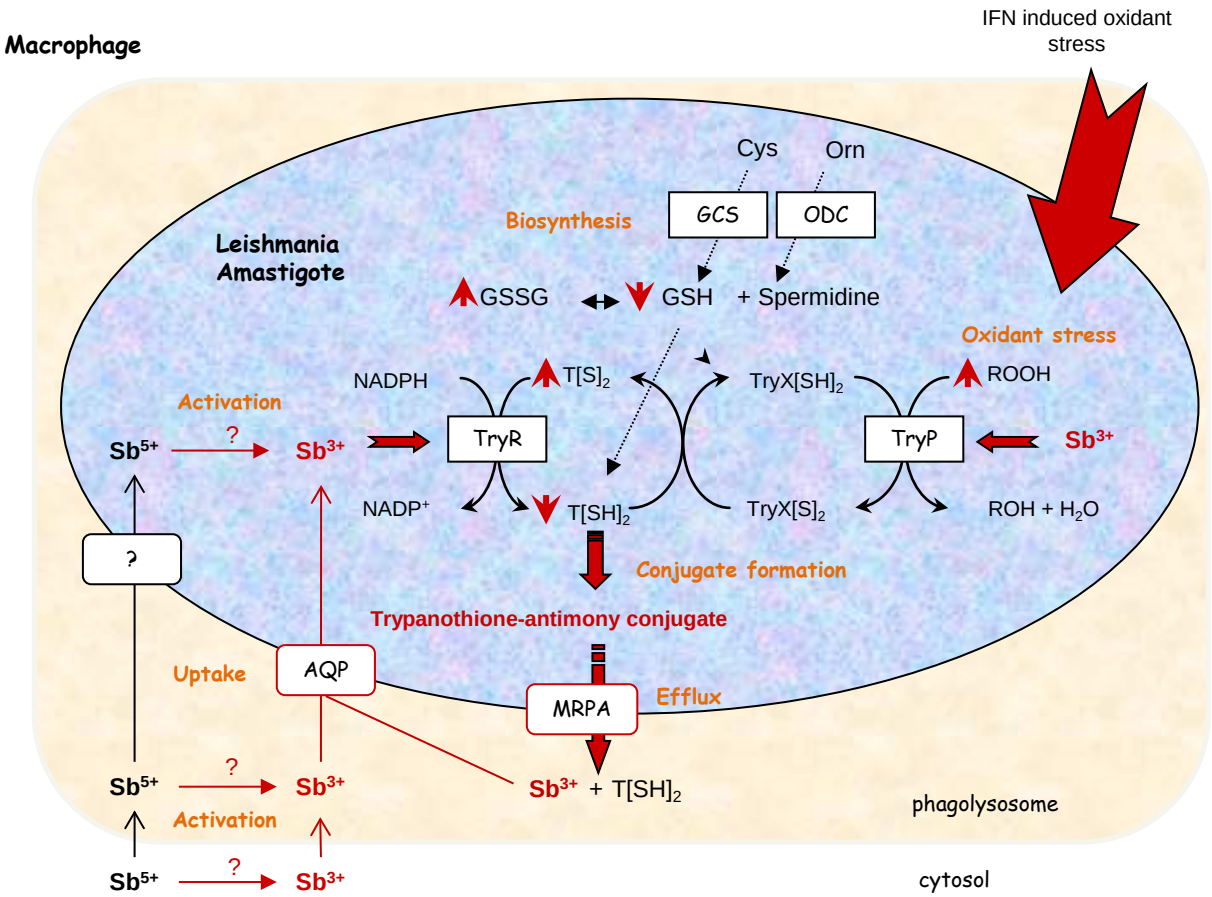


Figure 1.10 Postulated antimony mechanism of action (Section 1.2.4)

Sb^{V} is taken up by the macrophage and either reduced to Sb^{III} in the macrophage (Hansen *et al.*, 2011) or the amastigote (Shaked-Mishan *et al.*, 2001; Wyllie *et al.*, 2004). The transporter for Sb^{V} is unknown but Sb^{III} is taken up into the amastigote, as a glycerol mimic, via the aquaglyceroporin channel AQP1 (Gourbal *et al.*, 2004). The action of Sb^{III} is through inhibition of trypanothione reductase (TryR) and of the biosynthetic pathway of trypanothione ($T[SH]_2$) synthesis (Wyllie *et al.*, 2004). Sb^{III} forms a complex with $T[SH]_2$ and is effluxed out of the cell via the ATP binding cassette transporter MRPA (Legare *et al.*, 2001; El Fadili *et al.*, 2005). All of these actions lead to decreased levels of intracellular $T[SH]_2$, leaving the amastigote vulnerable to oxidative stress from the macrophage. This oxidative stress is additionally increased by Sb^{V} induced increased IFN-γ production (Murray *et al.*, 2000).

industry and other metal mining) or from antimony therapy (Sundar and Chakravarty, 2010).

At the current recommended therapeutic dose of 20 mg kg^{-1} day^{-1} for 30 days (Barrett, 2010), treatment with antimony is accompanied by many minor side effects including myalgia, arthralgia, electrocardiogram (ECG) changes and transient elevation in hepatic enzymes (Sundar *et al*., 1998). However, in up to 9% of cases serious cardiotoxicity can occur. Abnormal ECG changes progress to the life threatening arrhythmias of torsade de pointes and ventricular fibrillation therapy (Sundar and Chakravarty, 2010). The proportion of patients experiencing cardiotoxicity was higher prior to the introduction of IDA testing (Section 1.2.2). Pancreatitis, another potentially fatal adverse effect, is more common in HIV co-infected patients (Delgado *et al*., 1999). Regular monitoring of hepatic and pancreatic enzymes and ECG changes is recommended during therapy but this is not practical in the rural settings where antimony is often administered. Instead, antimony is not recommended for patients with co-morbidities (Barrett, 2010).

1.2.4 Antimony mechanism of action

The mechanism of action of pentavalent antimonials has not been fully elucidated despite almost a century of use. The toxic action has been established to be multifactorial with both direct and indirect actions and is mainly based around the thiol systems of the macrophage and parasite including the unique thiol of the kinetoplastid family 'trypanothione' ($T[SH]_2$) (Fairlamb *et al*., 1985; Wyllie *et al*., 2004). The mechanism of how antimony in its pentavalent form enters the amastigote and the exact mechanism of reduction to its trivalent form are still to be established. A summary of the mode of action of antimony can be found in Figure 1.10.

1.2.4.1 Direct action

Pentavalent antimonials are prodrugs which require to be reduced to trivalent form for their action. It is a remaining mystery, partly due to the technical difficulties of antimony speciation, as to where Sb^{V} is reduced to Sb^{III} – in the macrophage, in the amastigote or both? Both glutathione (GSH) and $T[SH]_2$, the latter more potently than the former due to the reducing activity of its dithiol structure, are able to non-enzymatically reduce Sb^{V} (Ferreira *et al*., 2003). GSH performs this optimally at pH 4.7-5.2 *in vitro* which, although this is not compatible with the neutral pH of the cytosol, could occur instead in the phagolysosome (Frezard *et al*., 2001). However, the predominant thiols in the phagolysosome are cysteine and cysteinyl glycine and not glutathione. A recent study found that the Sb^{V} treated uninfected macrophages contained up to 23% Sb^{III} (Hansen *et al*., 2011) although this analysis was performed in an acidic pH of 4.5 which may have led to an artificially elevated level of Sb^{III} through spontaneous reduction. $T[SH]_2$, found in the cytosol of the amastigote, reduces Sb^{V} at both pH 5 and 7, with a greater affinity than glutathione, resulting in formation of a complex of Sb^{III} and $T[SH]_2$ (Ferreira *et al*., 2003), (Yan *et al*., 2003).

The relative toxicity of Sb^{V} and Sb^{III} in different cell types, provides further evidence that reduction to toxic Sb^{III} may occur in the cytosol of the intracellular *Leishmania* amastigote. Whilst macrophages, are refractory to Sb^{V} at concentrations up to 1000 µg ml^{-1} (Wyllie *et al*., 2004), Sb^{V} has an EC_{50} against promastigotes of 8700 µg ml^{-1} (Roberts *et al*., 1995) and an axenic amastigote line was sensitive with an EC_{50} of just 200 µg ml^{-1} (Wyllie and Fairlamb, 2006a). This is in stark contrast to the similarities in sensitivity of the macrophage, promastigote and axenic amastigote to Sb^{III} with IC50s of 25, 11 and 8 µg ml^{-1}, respectively (Wyllie and Fairlamb, 2006a) (Wyllie *et al*., 2004).

Two thiol dependent enzymes have been suggested to catalyse antimony reduction. The first is thiol dependent reductase -1 (TDR1) whose presence was found to be 10-fold greater between amastigotes and promastigotes in a thiol enriched parasite lysate. This however does not fully account for the 60 to 600 fold difference in susceptibility between promastigotes and amastigotes (Roberts *et al*., 1995). The enzyme is thought to work on a spontaneously formed thiol metalloid compound rather than on the individual compounds (Denton *et al*., 2004). Although its structure has been solved (Fyfe *et al*., 2012) fuller experimental evidence is required to demonstrate this enzyme is definitively responsible for Sb^{V} reduction.

The other putative reductase was identified through working from the similarity between the metalloids arsenic and antimony: the sequence of an arsenate reductase, ScAcr2p from *Saccharomyces cerevisiae*, was used to identify a closely related *L. major* enzyme, LmACR2 (Zhou *et al*., 2004). Transfection of this enzyme into promastigotes conferred increased sensitivity to Sb^{V} in the differentiated intracellular amastigote form showing that it may have an activating role. Further studies demonstrate that this enzyme is also involved in the phosphotyrosine pathway (Zhou *et al*., 2006).

1.2.4.2 Transport

It has been known for over 30 years that antimony accumulates within the *Leishmania* parasite (Croft *et al*., 1981). More recent studies, using inductively coupled plasma mass spectrometry (ICP-MS) which allows for speciation, have been able to demonstrate that both Sb^{V} and Sb^{III} enter the parasite via different routes (Brochu *et al*., 2003) and that reduction from Sb^{V} and Sb^{III} occurs within the amastigote (Shaked-Mishan *et al*., 2001). Furthermore, there is a greater accumulation of antimony into the amastigote than the promastigote form (Shaked-Mishan *et al*., 2001).

The mechanism of entry of trivalent metalloids into *Leishmania* has been established as the aquaglyceroporin (AQP) channel through transfection of AQP1 into promastigotes of *L.tarentolae, L.major* and *L.infantum* leading to increased production, accumulation and sensitivity to Sb^{III} (Gourbal *et al*., 2004). The transporter for entry of Sb^{V} has not as yet been identified.

1.2.4.3 Thiol perturbation

Once Sb^{III} is inside the amastigote it is thought to exert its toxic effect by 2 mechanisms. When axenic amastigotes are incubated with Sb^{III} there is a time dependent decrease in intracellular thiols (Wyllie *et al*., 2004) which can be detected in the supernatant. The Sb^{III} forms a complex with either $T[SH]_2$ or GSH and is effluxed from the amastigote into a vacuole or into the phagolysosome via PgpA transporters (Dey *et al*., 1996) including MRPA (El Fadili *et al*., 2005; Légaré *et al*., 2001). The inhibition of trypanothione reductase leads to an accumulation of the disulphide forms of trypanothione and glutathione, which combined with the efflux leads to a reduction in the thiol buffering capacity of the leishmania parasite and adversely affects the redox potential (Wyllie *et al*., 2004). This oxidising environment induces amastigote apoptosis and nuclear fragmentation – a mode of cell death that has been established to occur (Sereno *et al*., 2001) (Sudhandiran and Shaha, 2003).

1.2.4.4 Action on immune system

As well its direct toxic effect antimonials have an essential indirect mode of action via the immune system. Pentavalent antimonials are profoundly less effective in both T-cell deficient BALB/c mice (Murray *et al*., 1989) and severe combined immune deficiency (*scid*) mice (Escobar *et al*., 2001). Additionally Sb^{V} has an inhibitory effect on the tyrosine phosphatase SHP-1 (Pathak and Yi, 2001) leading to increased IL-12 and IFN-γ (Murray *et*

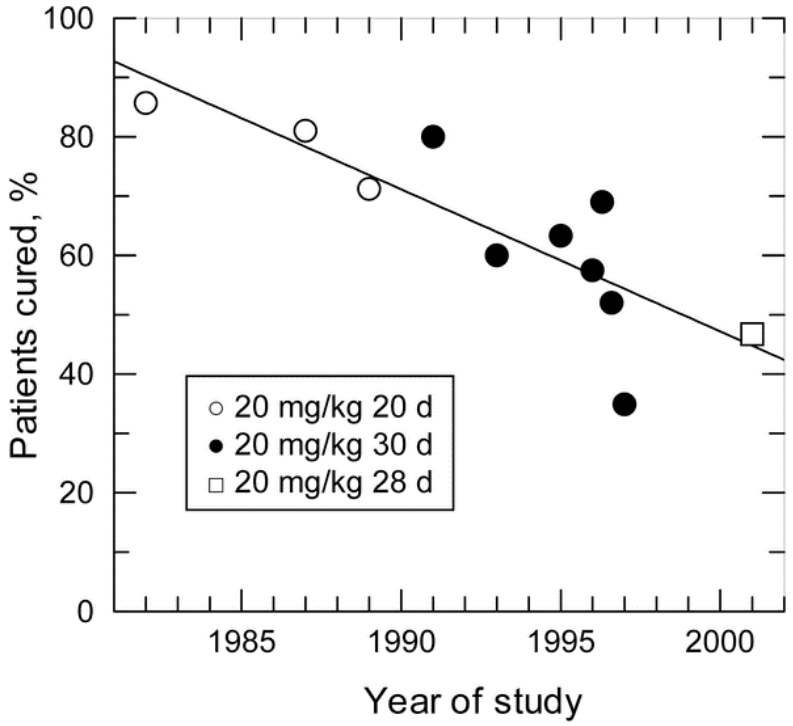


Figure 1.11 Decline in efficacy of antimonials at 20 mg kg^{-1} in Bihar: results of clinical studies between 1980 and 2004

The graph is data extracted from a table in (Guerin *et al* 2002). Doses regimes are indicated in the figure key.

Linear regression correlation coefficient = -0.83. (Perry *et al.*, 2011)

al., 2000). This and other effects on protein kinase signalling result in increased production of reactive oxygen and nitrogen species which ultimately lead to *Leishmania* cell death (Mookerjee *et al*., 2006; Rais *et al*., 2000).

1.2.5 Resistance development and mechanisms

Chemotherapy with pentavalent antimonials was effective in all regions of the world until the 1990s despite inherent interspecies differences in antimonial susceptibility (Allen and Neal, 1989). A decrease in treatment efficacy was first noted when VL re-emerged in India in the late 1970s following its near eradication as a secondary effect of a National Malarial Eradication programme in the 1950s and 60s (Peters, 1981). On re-emergence, the prior 6 day regime was found to be 'grossly inadequate' (Thakur, 1984) and was increased to 10 mg kg^{-1} for 20 days, and then to 20 mg kg^{-1} for 30 days over the next 20 years, in an effort to retain efficacy of this drug which was the sole established treatment for VL at this time. However despite this, there were reports of declining cure rates from the field (Sundar *et al*., 2000) and in the context of clinical trials (Olliaro *et al*., 2005). By the end of the twentieth century the cure rate was less than 1 in 2 (Figure 1.11).

The reason for the dramatic development of treatment failure in India was thought to be the misuse of antimony in the under-regulated private health care sector. In 1994 a study reported that 227/312 (73%) of patients obtained treatment from an unqualified medical practitioner. Two hundred and twenty five (72%) did not complete a treatment course that complied with the current WHO guidelines, 132 (42%) did not take it regularly and 112 (36%) stopped on their own initiative (Sundar *et al*., 1994). All of the above situations could lead to sub therapeutic delivery of SSG and allow for the development of resistance.

Although parasitologists had been inducing resistance to antimonials using trivalent metalloids and laboratory parasite strains since clinical failure was reported (Detke *et al*., 1989), it was not until 1998 that SSG resistance in clinical isolates was reported that was related to clinical response. This study showed, although the numbers are small, a high correlation between SSG susceptibility of the amastigote in an *in vitro* intracellular environment and clinical outcome from SSG treatment (Lira *et al*., 1999). Since then, although many studies have been performed on resistant and sensitive clinical isolates, the correlation between SSG susceptibility and clinical response has been called into question (Rijal *et al*., 2007).

1.2.5.1 Selection of Leishmania parasite resistance

This section will look at the molecular mechanisms of SSG resistance in *L.donovani* and discuss how these relate to findings in clinical isolates and to clinical failure *per se*.

Mechanisms of parasite antimony resistance have now been being investigated for over 30 years and have been found to be multifactorial including drug metabolism, drug influx, membrane transport and sequestration, thiol metabolism, genome plasticity, programmed cell death and manipulation of macrophage functions (Croft *et al*., 2006). It is important to note the variability in methods that have been used to investigate drug resistance:

- Parasite species: Antimonial resistance has been extensively studied in *L.tarentolae*, a *Leishmania* species found in the white-spotted wall gecko (*Tarentola annularis*) that is non-pathogenic to humans (Brochu *et al*., 2004; Dey *et al*., 1994; Dey *et al*., 1996; Guimond *et al*., 2003; Haimeur *et al*., 2000; Légaré *et al*., 1997; Mukhopadhyay *et al*., 1996). The work on this species, although it has identified many common mechanisms has also identified differences between *L.tarentolae* and

the *Leishmania* species relevant to humans. For example the level of trypanothione is 50 fold greater in *L.infantum* than *L.tarentolae* (El Fadili *et al*., 2005). Interestingly, the genome of *L.tarentolae* has recently been assembled and, although there is a high degree of synteny, the species is missing many genes relating to the pathogenic intracellular amastigote stage of the life cycle (Raymond *et al*., 2012).

- Parasite phenotype: Resistance has been studied in promastigote, axenic amastigote and intracellular amastigote forms (Croft *et al*., 2006).
- Method of resistance development: As well as selecting for resistance with Sb^{III} and Sb^{V}, in laboratory strains resistance has been selected using As^{III} because selection for resistance to As^{III} also leads to cross resistance to Sb^{III} (Brochu *et al*., 2004; Dey *et al*., 1994; Dey *et al*., 1996; Guimond *et al*., 2003; Haimeur *et al*., 2000; Légaré *et al*., 1997; Mukhopadhyay *et al*., 1996). Some Sb^{V} preparations used in early studies were subsequently discovered to contain the preservative chlorocresol which has its own leishmanicidal action (Ephros *et al*., 1997), thus resistance was selected for to the chlorocresol as well as to Sb^{V} itself. The final method of resistance development is within the patient: clinical isolates from patients and dogs have been extensively studied both before and after exposure to Sb^{V} treatment (Decuypere *et al*., 2005; Faraut-Gambarelli *et al*., 1997; Gramiccia *et al*., 1992).

1.2.5.2 Loss of Sb^{V} activation as a resistance mechanism

Inhibition of the activation of Sb^{V} by reduction to Sb^{III} has been proposed to be a mechanism of Sb^{V} resistance based on studies that demonstrated the lack of toxicity of Sb^{V} against a Sb^{III} generated resistant axenic amastigote line compared with control (Shaked-Mishan *et al*., 2001). Although transfection of the proposed Sb^{V} activator, *Lm*ACR2, led to

increased sensitivity to sodium stibogluconate in intracellular amastigotes (Zhou *et al*., 2004), no evidence of changes in expression levels of this enzyme or the other putative reductase, TDR1, were identified in a study of resistant and sensitive clinical isolates in Nepal (Decuypere *et al*., 2005).

1.2.5.3 Changes in transport of Sb^{III} as a resistance mechanism

Evidence for the involvement of drug influx in antimonial resistance was demonstrated by the overexpression of *Lm*AQP1 into both selected for and natural resistant strains leading to increased susceptibility to both Sb^{III} and As^{III} as promastigotes and Sb^{V} as amastigotes within macrophages (Gourbal *et al*., 2004). Additionally an induced point mutation in *Lm*AQP1 led to marked decrease in susceptibility to Sb^{III} and As^{III} in promastigotes (Uzcategui *et al*., 2008).

Analysis of antimony sensitive and resistant field isolates only weakly support AQP1 as a mechanism for antimonial resistance with contradictory results. Analysis of 14 field isolates (5 sensitive and 9 resistant) from VL and PKDL patients in Bihar (Mandal *et al*., 2010) showed good correlation between accumulation of Sb^{III} and AQP1 expression levels and antimonial resistance. Gene expression analysis of Nepalese isolates (2 sensitive and 2 resistant) showed a lower expression of AQP1 in resistant strains (Decuypere *et al*., 2005). However, in another study from India, although there was evidence of decreased accumulation of Sb^{III}, analysis of 6 clinical isolates (2 sensitive and 4 resistant) showed no correlation between AQP1 expression levels and antimonial resistance and an increased copy number of AQP1 in resistant isolates (Maharjan *et al*., 2008).

The importance of ATP-dependent efflux pumps of PgpA in conferring resistance to metalloids in micro-organisms has been well researched (Cervantes *et al*., 1994). The

MRPA metalloid thiol efflux pump has been implicated in antimonial resistance in both laboratory generated resistance (El Fadili *et al*., 2005; Grondin *et al*., 1997; Guimond *et al*., 2003; Haimeur *et al*., 2000) and as an extra chromosomal amplification in natural resistant isolates (Brotherton *et al*., 2013; Leprohon *et al*., 2009; Mukherjee *et al*., 2007)). Transfection of MRPA into *L.infantum* confers high level antimonial resistance in both axenic and intracellular amastigotes (El Fadili *et al*., 2005): the Sb-thiol conjugate is thought to be sequestered inside a vacuole or extruded from the parasite. Of note, macrophages infected with antimonial resistant isolates also upregulate their MRP1 and PgpA transporters in their host cells (Mookerjee *et al*., 2008).

1.2.5.4 Upregulation of thiol synthesis in leishmania as a resistance mechanism

The result of upregulation of MRP transporters is low levels of intracellular metalloid decreasing the toxic effect on the leishmania parasite. However, overexpression of the MRPA means that protective thiols are being pumped out of the amastigote. Resistant parasites have higher levels of the protective intracellular trypanothione than sensitive parasites (Mandal *et al*., 2007) – this is achieved by upregulation of the biosynthetic pathways and maintaining trypanothione in a reduced form. Elevated levels of trypanothione reductase (TR), ornithine decarboxylase (ODC) and gamma-glutamyl cysteine synthetase (γ-GCS) are implicated in clinical isolates from India (Mittal *et al*., 2007; Mukherjee *et al*., 2007), TR and ODC in Nepal (Decuypere *et al*., 2005) and TR and ODC in *L.braziliensis* in Brazil (Adaui *et al*., 2011). This high intracellular thiol level is likely to be partially responsible for increased tolerance to nitric oxide (NO) (Holzmuller *et al*., 2005) and hydrogen peroxide (H_2O_2) (Carter *et al*., 2005). However, upregulation of tryparedoxin peroxidase to reduce levels of reactive oxygen species has also been identified

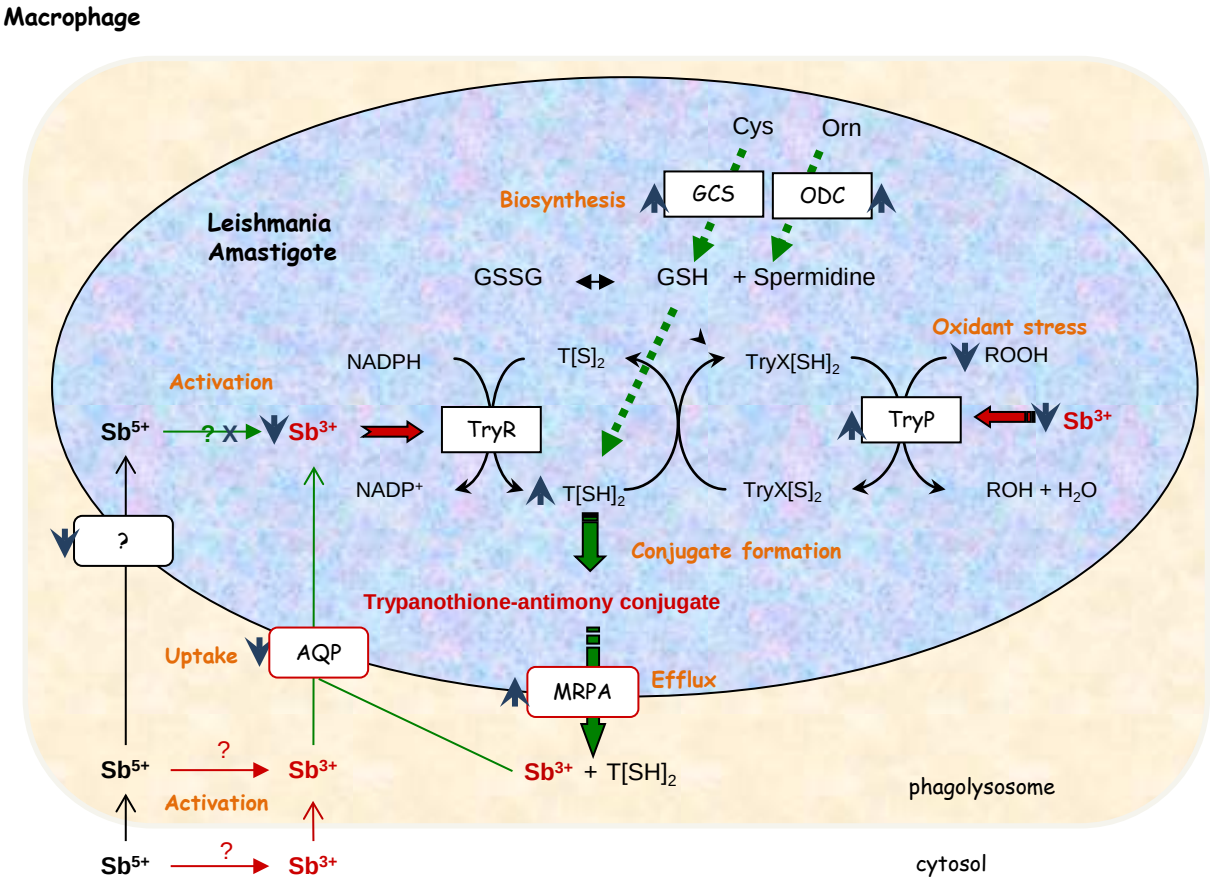


Figure 1.12 Postulated antimony mechanisms of resistance (Section 1.2.5)

The changes in metabolic state of the resistant leishmania amastigote are depicted in blue. Established mechanisms of resistance include downregulation of the influx pump aquaglyceroporin (AQP), upregulation of the efflux pump MRPA, and upregulation of trypanothione peroxidase (TryP) and enzymes of trypanothione biosynthesis (ornithine decarboxylase, ODC and gamma glutamylcysteine synthetase, γ-GCS) . This results in elevated levels of thiols within the amastigote, increasing it's defences against oxidative stress. Other proposed mechanisms of resistance include downregluation/loss of the Sb^{V} transporter and downregulation/loss of the activation step from Sb^{V} to Sb^{III}. Reviewed in (Ait-Oudhia *et al*., 2011).

in laboratory generated antimonial resistant *L.tarentolae* (Wyllie *et al*., 2008) and field isolates (Wyllie *et al*., 2010).

1.2.5.5 Manipulation of the immune system as a mechanism of resistance

As well as ensuring high levels of protective intracellular thiols antimonial resistant *Leishmania* have also been shown *in vitro* to manipulate the host immune response with inhibition of the PI3 kinase/AKT and NFκβ pathways (Haldar *et al*., 2010) and increased levels of IL-10 (Haldar *et al*., 2010; Mukhopadhyay *et al*., 2011). Patients who did not respond to SSG therapy were more likely to have a TH2 response to VL infection with low levels of IFN-γ (Thakur *et al*., 2003). Whether this immune reaction is parasite or host driven has not yet been elucidated.

1.2.5.6 Additional mechanisms of resistance

Additional reported mechanisms involve changes in membrane fluidity and over expression of terminal glycoconjugates (Mukhopadhyay *et al*., 2011). Overexpression of heat shock protein has also been implicated (Brochu *et al*., 2004). Use of proteomic techniques (Brotherton *et al*., 2013; El Fadili *et al*., 2009; Kaur and Rajput, 2014) such as stable isotope labelling of amino acids in culture (SILAC) has confirmed known resistance mechanisms and will hopefully identify new ones. However care needs to be taken on what phenotype of *Leishmania* resistance is being generated and analysed.

1.2.5.7 Summary

There is heterogeneity in resistance mechanisms but a schematic overview of the resistant parasite is illustrated in Figure 1.12. Once parasites have become resistant to metalloids the resistance is mainly thought to be stable even in the absence of metalloid pressure (El Fadili *et al*., 2005; Grogl *et al*., 1989) and in fact may have a selective advantage over sensitive

parasites with increased metacyclogenesis, infectivity and parasite growth within hosts (Vanaerschot *et al*., 2011; Vanaerschot *et al*., 2013; Vanaerschot *et al*., 2010). The ultimate goal is to find a parasitic marker that correlates highly with patient treatment failure that could act as a clinical test for resistance at the bedside pre-treatment.

1.3 Arsenic

1.3.1 Description of metalloid

Arsenic is a chemical element with the symbol As, belonging to the metalloids and Group 15 of the Periodic Table of Elements (Figure 1.9) and (Table 1.3)(Sekhon, 2013). Arsenic's main use is to strengthen copper and lead alloys (e.g. in a car battery). It is also used in semiconductors, herbicides, pesticides and insecticides as well as medicines (Section 1.3.3). It is an important ingredient found in flypaper, cosmetics, glass, ceramic enamels, paints, metallurgy, tanning, taxidermy and in candles (Doyle, 2009).

Arsenic can exist in oxidation states of +5, +3 and 0. It readily forms covalent bonds with hydrogen, oxygen and carbon and alloys with metals. The pentavalent arsenates, in the form of H_3AsO_4, act as phosphate mimics, are taken up into cells by phosphate transporters and interfere with energy metabolism. Trivalent arsenic compounds are derived from arsenite $H_2As_2O_3$ and arsenic trioxide As_2O_3 and are more toxic than pentavalent compounds. Arsenic has a strong affinity to sulphur which predicts its accumulation in body organs (Ho, 2005).

1.3.2 Arsenic metabolism

Humans readily absorb arsenic through their gastrointestinal system and metabolise the inorganic forms via a series of reduction and methylation reactions leading to the

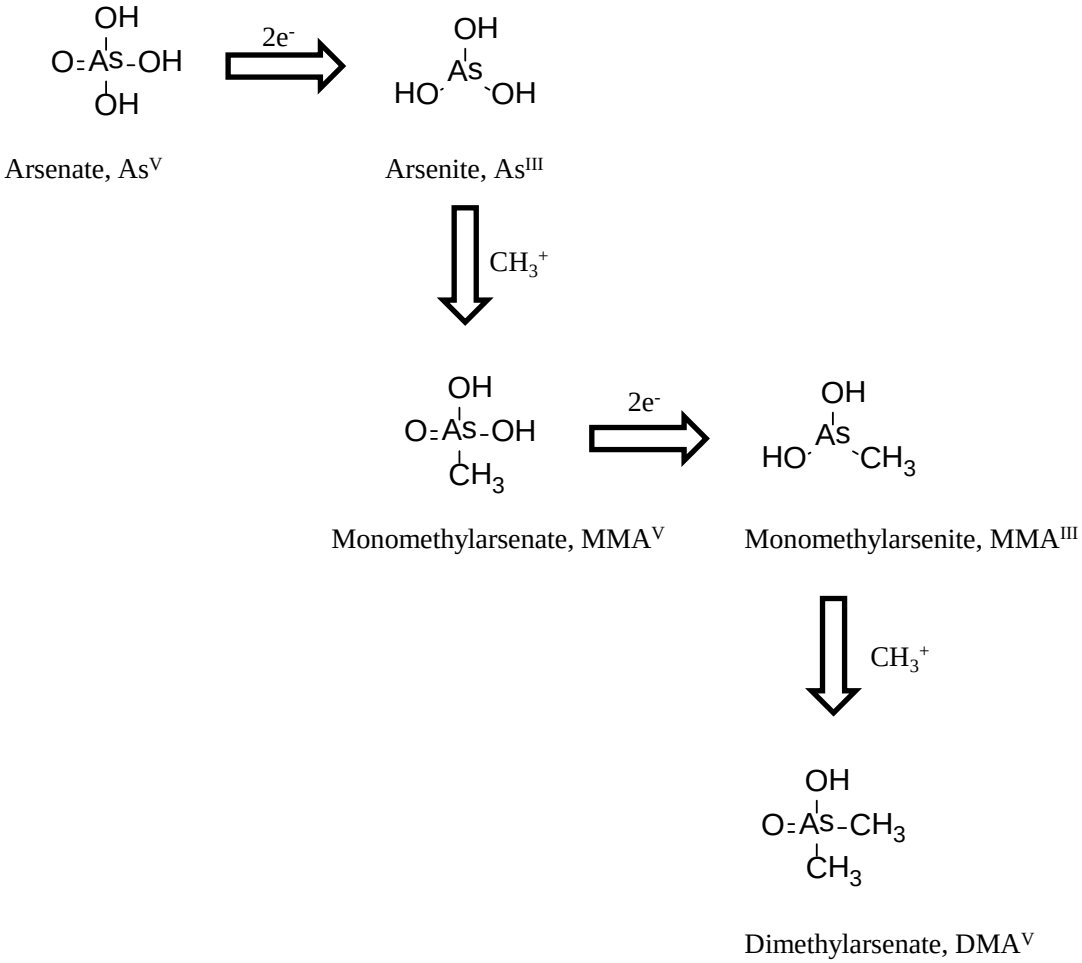


Figure 1.13 Human metabolism of arsenic Arsenic is metabolised via a series of reduction and methylation reactions. The main site of methylation is the liver although most organs are thought to have some methylation capacity. The majority of ingested arsenic is excreted in the urine within 48 hours of ingestion in the chemical forms of 10–30% inorganic arsenic, 10-20% MMA, 60-80% DMA (Vahter *et al.*, 2001)

pentavalent compound dimethylarsenic acid (DMA) (Figure 1.13). Following bioactivation from As^{V} to As^{III}, As^{III} is thought to bind to dithiol groups before methylation (Vahter and Concha, 2001). Arsenic (+3 oxidation state) methyltransferase (AS3MT) (Lin *et al*., 2002) is able to methylate both As^{III} and monomethylarsenic acid (MMA) but has a different affinity for the 2 substrates (Engstrom *et al*., 2013). Methyltransferases can be inhibited by excess substrate. The main site of methylation is the liver but most tissues have methylation capacity (Vahter, 2002).

A decreased methylation capacity leads to increased tissue concentrations of arsenic. Methylation capacity is influenced by age, gender, ethnicity, dose, pregnancy, nutrition and genetic polymorphism. However in general, arsenic in urine consists of 10–30% inorganic arsenic, 10–20% MMA, and 60–80% DMA (Vahter, 2002). Although methylation was initially viewed as a detoxification mechanism, and indeed the pentavalent methylated species have lower affinity for sulfhydryl groups, lower cellular uptake and higher excretion, the trivalent mono methylated species is the most toxic (Styblo *et al*., 2000) and is thought to play a key role in the carcinogenicity of arsenic (Kojima *et al*., 2009). Mechanisms other than methylation (e.g. protein binding) are likely to be important in detoxification of inorganic arsenic (Vahter, 2002).

Although the majority of arsenic is excreted within 48 hours of ingestion, accumulation has been observed in hair, nails, skin (Vahter and Concha, 2001) and liver (Mazumder, 2005) in humans. In animal studies arsenic levels in organs are related to dose and duration of exposure (Das *et al*., 2005). However the relationship is more complex in humans, likely due to differences in methylation capacity and membrane transporters between and within population groups (Drobna *et al*., 2010; Melak *et al*., 2014).

1.3.3 History of therapeutic use

Although infamous as a poison, arsenic also has a long history of use as a treatment. The therapeutic properties of arsenic in the form of orpiment (As_2S_3) and realgar (As_2S) have been utilised for more than 3000 years in ancient Chinese and Hindu medical practices and the tonic 'arseniko' was described by Hippocrates (469-377 BC) (Doyle, 2009). Arsenic compounds were successfully used internally by Paracelsus in the 15th Century AD. Dr Thomas Fowler (1736-1801) developed 1% potassium arsenite ($KAsO_2$, Fowler's solution) which was widely used throughout the 19th century for many different ailments including goitre, Hodgkin's disease and Herpes Zoster. It remained on the US pharmacopeia as late as 1950 (Kritharis *et al*., 2013).

Paul Ehrlich (1854-1915) was responsible for producing the drug arsphenamine which was found to be effective against syphilis. This led to the effective use of the arsenic compound salvarsan against syphilis and yaws (Doyle, 2009) and the development of the compound melarsoprol for trypanosomiasis (Friedheim, 1951; Friedheim, 1949). Over the course of the 20th century, arsenic was replaced by penicillin in the treatment of syphilis but its use of arsenic as an anti-cancer agent re-emerged. Arsenic trioxide (ATO) is now fully licenced by the FDA as a treatment for relapsed acute promyelocytic leukaemia (APML). ATO is undergoing trials as chemotherapy for many different solid tumours (Kritharis *et al*., 2013) and recent results suggest its place may be in combination with other agents (Subbarayan and Ardalan, 2014).

The effect on arsenic on cellular metabolism is diverse, including actions within the mitochondria, endoplasmic reticulum and nuclear bodies (found in the nuclei of malignant mammalian cells) (Ho, 2005; Kritharis *et al*., 2013). Its therapeutic effect in APML is

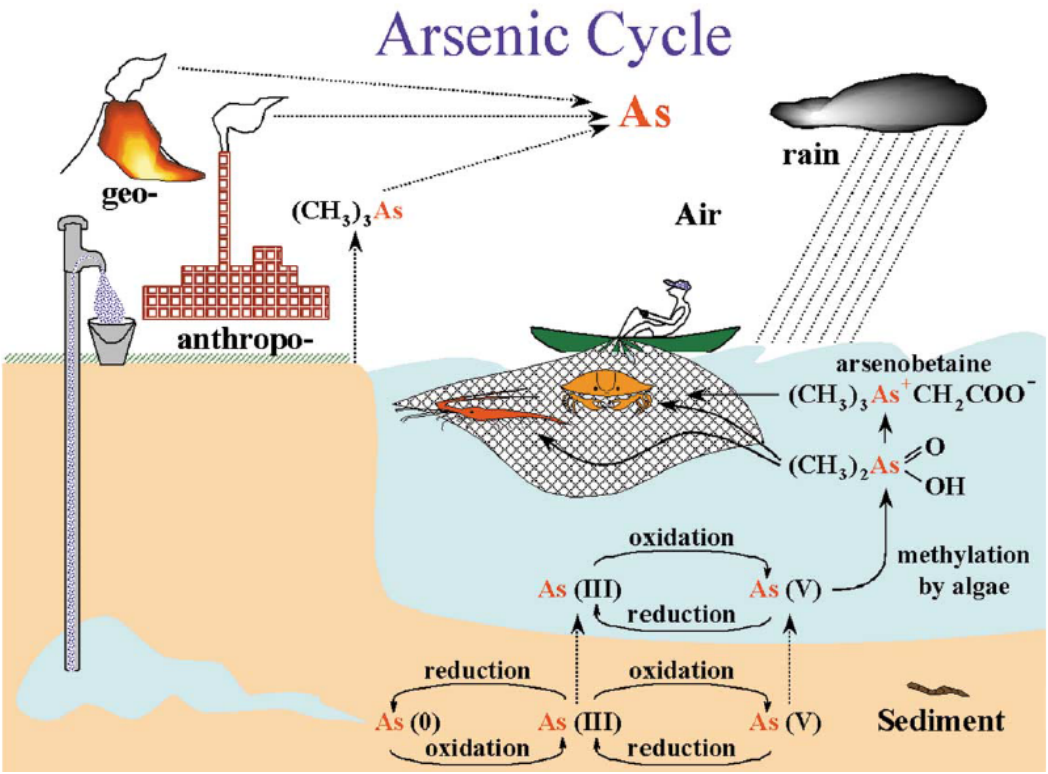


Figure 1.14 Arsenic global geocycle

Figure from (Mukhopadhyay *et al.*, 2002)

thought to be secondary to arsenic-induced degradation of all PML isoforms which is dependent on the ubiquitin E3 ligase RNF4 (Hands *et al*., 2014).

Reported toxic effects from pharmacological doses of arsenic include gastrointestinal effects, flushing, fatigue, dysesthesias and dizziness. QT prolongation and ventricular tachycardia, neutropenia, thrombocytopenia, hyperglycemia, hepatic enzyme elevations can be observed. There have only been 2 cases reported of fatal encephalopathy with arsenic trioxide in Taiwan at 0.24 mg kg^{-1} day^{-1} (Subbarayan and Ardalan, 2014). The higher dose required for the arsenical based drug melarsoprol, still in use for HAT in some regions (Kuepfer *et al*., 2012), has led to fatal encephalopathy in more than 8% of patients.

1.3.4 Arsenic contamination of water

Arsenic is present all around us and forms a geocycle that man takes part in (Figure 1.14) (Mukhopadhyay *et al*., 2002). The main source of arsenic is igneous. Arsenic is widespread in the upper part of earth's crust with an average concentration in seawater of 2.6 ppb, fresh water 0.4 ppb, atmospheric dust of 50 – 400 ppm and soil 0.1 – >1000 ppm kg^{-1} (Mukhopadhyay *et al*., 2002).

1.3.4.1 Discovery and epidemiology of arsenic contamination

Although an abundant natural element, the occurrence of arsenic in natural waters has only been known about for just over 100 years (Ravenscroft *et al*., 2009). The first recorded arsenic measurement in water was by the German chemist Fresnius at Wiesbaden Spa in 1885 and the first recorded adverse health effect was skin cancer in Poland in 1898 (Mandal and Suzuki, 2002). Despite an endemic outbreak in Argentina in the 1920s, arsenic contamination of water and its impact on health remained largely unknown until the latter

part of the 20th Century when publications on ‘blackfoot disease’ emerged from Taiwan (Chen *et al*., 1985; Tseng *et al*., 1968).

In tropical Asia in the 1970s there was an accelerated use of groundwater through the drilling of tens of millions of shallow tube wells to provide clean, microbiologically safe sources of drinking water. This policy was promoted by the United Nations Children’s Fund (UNICEF) and supported by the World Bank due to high under five diarrhoeal mortality in these countries, secondary to bacterial pollution of shallow, open dug wells leading to outbreaks of dysentery and cholera (Meharg, 2005). Tube wells are closed systems in which a stainless steel pipe covered with a strainer accesses water from an underground aquifer which is drawn up to the surface through the use of a hand pump. In large geographical areas, these shallow tube wells accessed arsenic contaminated ground water leading to the most extensive case of arsenic poisoning in world history (Ravenscroft *et al*., 2009). That arsenic is colourless, odourless and tasteless catastrophically delayed the discovery of its presence in drinking water.

The occurrence of arsenic in groundwater was established as an issue in the US in 1988 (Welch *et al*., 2000). Although the first report from West Bengal was in 1983, it was not until a publication which described the extent of poisoning in six districts of West Bengal and political pressure exerted Dipankar Chakraborti at the School of Environmental Studies that the issue was brought to the international media including CNN and BBC (Ravenscroft *et al*., 2009). This prompted widespread testing in neighbouring Bangladesh and extensive arsenic pollution was also discovered here and in the river basins of Nepal, Myanmar, Cambodia, Vietnam and Pakistan (Nordstrom, 2002). Following the turn of the 20th Century arsenic was detected upstream of the Ganges in Bihar (Chakraborti *et al*., 2003) and Uttar Pradesh (Ahamed *et al*., 2006) and extensively mapped in Chinese

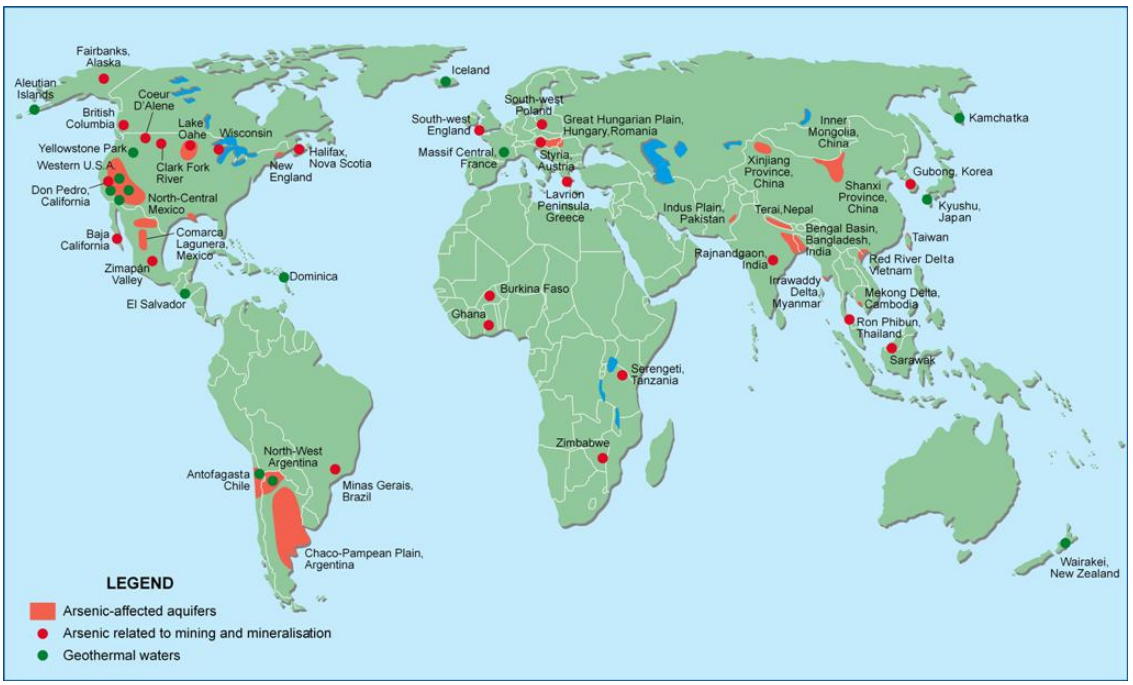


Figure 1.15 Worldwide distribution of arsenic in groundwater

Geographical representation of documented problems with arsenic in groundwater (>50 µg l^{-1}). Source: British Geological Survey, updated from Smedley and Kinniburgh 2002.

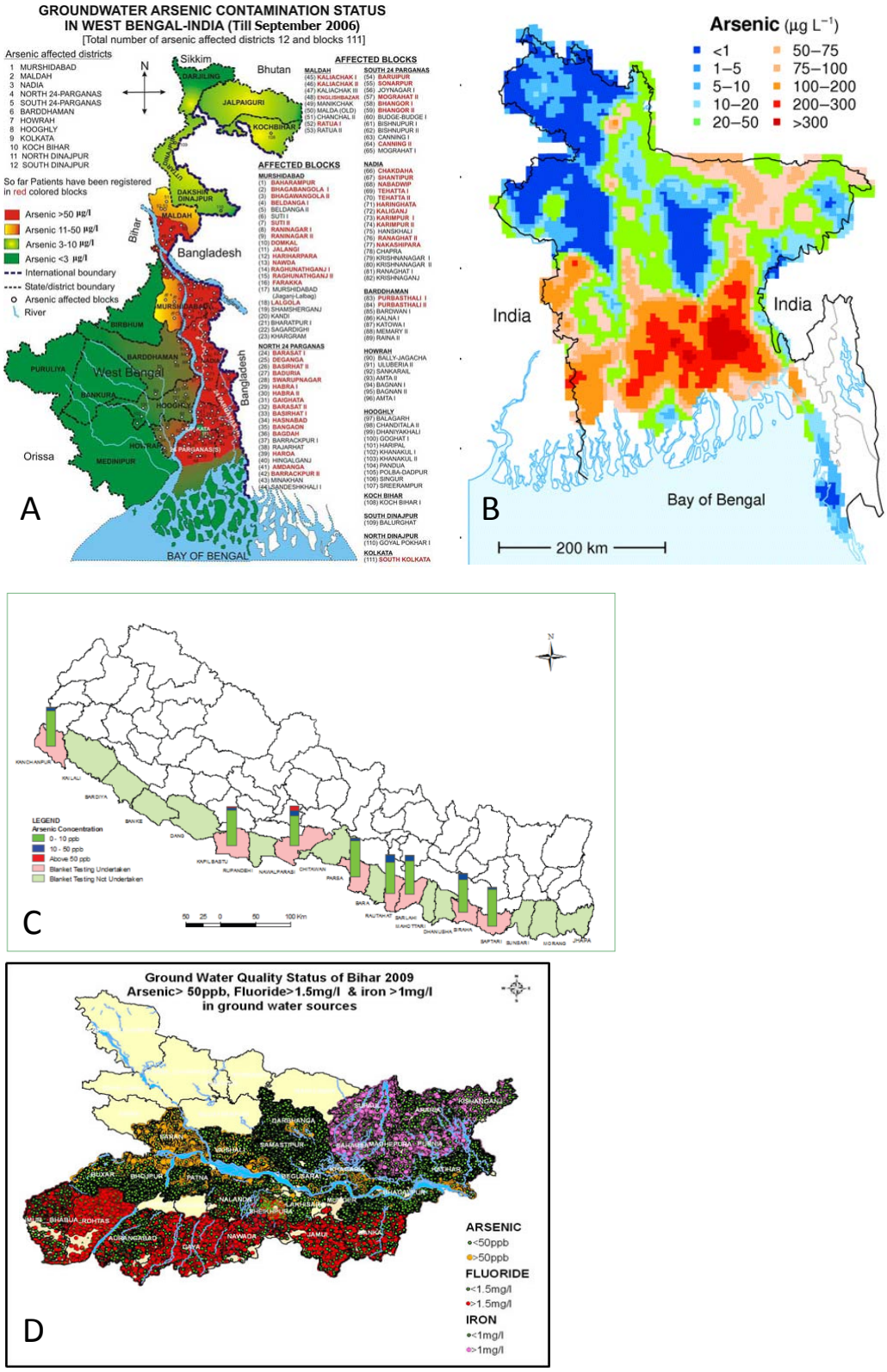


Figure 1.16 Distribution of arsenic contamination in West Bengal, Bangladesh, Nepal and Bihar

Sources: West Bengal; School of Environmental Studies, Jadvapur University (Panel A); Bangladesh: British Geological Survey (Panel B); Nepal: Genesis report, 2005 (Panel C); Bihar: Public Health and Education Department (Panel D).

Table 1.4 Worldwide distribution of arsenic

Source: Adapted from (Nordstrom *et al.*, 2002)

Country/region	Concentration ($\mu g\ l^{-1}$)	Potential exposed population	Environmental conditions
Bangladesh	< 1 to 2,500	30,000,000	Natural; alluvial/deltaic sediments with high phosphate,* organics
West Bengal, India	< 10 to 3,200	6,000,000	Similar to Bangladesh
Vietnam	1 to 3,050	> 1,000,000	Natural; alluvial sediments
Thailand	1 to > 5,000	15,000	Anthropogenic; mining and dredged alluvium
Taiwan†	10 to 1,820	100,000 to 200,000	Natural; coastal zones, black shales
Inner Mongolia	< 1 to 2,400	100,000 to 600,000	Natural; alluvial and lake sediments; high alkalinity
Xinjiang, Shanxi	40 to 750	> 500	Natural; alluvial sediments
Argentina	> 1 to 9,900	2,000,000	Natural; loess and volcanic rocks, thermal springs; high alkalinity
Chile‡	100 to 1,000	400,000	Natural and anthropogenic volcanogenic sediments; closed basin; lakes, thermal springs, mining
Bolivia§	-	50,000	Natural; similar to Chile and parts of Argentina
Brazil‖	0.4 to 350	-	Gold mining
Mexico	8 to 620	400,000	Natural and anthropogenic; volcanic sediments, mining
Germany	< 10 to 150	-	Natural: mineralized sandstone
Hungary, Romania	< 2 to 176	400,000	Natural; alluvial sediments; organics
Spain¶	< 1 to 100	> 50,000	Natural; alluvial sediments
Greece#	-	150,000	Natural and anthropogenic; thermal springs and mining
United Kingdom**	< 1 to 80	-	Mining; southwest England
Ghana	< 1 to 175	< 100,000	Anthropogenic and natural; gold mining
USA and Canada	< 1 to > 100,000	-	Natural and anthropogenic; mining, pesticides, As_2O_3 stockpiles, thermal springs, alluvial, closed basin lakes, various rocks

provinces. Although arsenic contamination can be found throughout the world, Asia is thought to be the worst effected with likely more than 100 million people drinking water with arsenic levels greater than the WHO recommendation of $\geq$10 μg L^{-1} (Sen and Biswas, 2013). Figure 1.15 and Table 1.4 give a summary of the global distribution of arsenic contamination.

In the Indian subcontinent the River Ganges exits the Himalayas in Northern India and runs through the states of Uttar Pradesh, Bihar, Jharkland and West Bengal before joining with the Brahmaputra in Bangladesh where the highest levels of arsenic contamination are found (Argos *et al*., 2010). Figure 1.16 shows the relative distribution of arsenic contamination in Nepal, Bangladesh and the worst affected states of India: West Bengal and Bihar. Importantly, the Bangladesh, Nepal and West Bengal maps represent exhaustive testing of every tube well in affected areas whereas the map of Bihar only represents surveys where approximately 10% of the wells were sampled with no sampling having been performed in some areas. In Bihar this is mainly due to a priority to sample within 10 km of the River Ganga (Pi *et al*., 2000).

1.3.4.2 Natural causes of arsenic contamination

In the Earth's crust the most important accumulations of arsenic are found associated with sulphides and oxides. In the Indian subcontinent the arsenic is present in subsurface water as a result of rock weathering, downstream transport and sediment deposition of arsenic rich minerals originally present in the Himalayas leading to a concentration of arsenic contaminated groundwater in the tropical river basins of Asia (Nickson *et al*., 1998).

Release of arsenic into water occurs where neither sulphides nor oxides can remove arsenic from solution (Ravenscroft *et al*., 2009). The four mechanisms of this are listed

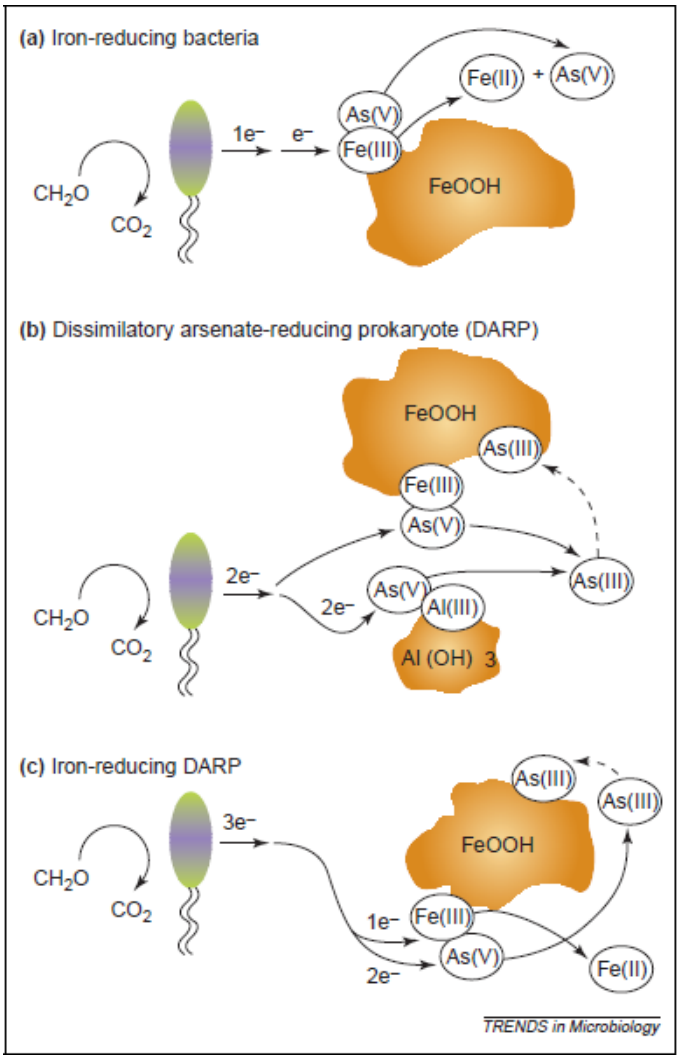


Figure 1.17 Three mechanisms of arsenic mobilisation by metal reducing bacteria

The micro-organisms oxidise organic matter and use As^{V} and Fe^{III} as their terminal electron acceptor. It is possible that all these mechanisms can occur simultaneously in the same aquifer. Figure from (Oremland and Stoltz, 2005)

below. Reductive dissolution is the mechanism which occurs in the Indian subcontinent and the one which will be focussed on in this study.

1. Reductive dissolution: Arsenic is often found adsorbed onto the surface of iron oxides, which are the most important minerals for controlling the occurrence of arsenic in groundwater. When iron oxides are broken down and dissolve under the influence of oxygen consuming decaying organic matter, arsenic is released (Oremland and Stolz, 2005). The groundwater produced is strongly reducing and contains high levels of iron and bicarbonate. Characteristically nitrate and sulphate are absent. Figure 1.17 show the action of dissimilatory arsenic reducing prokaryotes (DARPs) which respire using arsenate's similarity to phosphate producing As^{III} (Oremland and Stolz, 2005).
2. Alkali desorption: This occurs in the presence of oxygen, nitrogen or sulphate, at high pH ≥8.0 and produces alkali-oxic water typically with low levels of iron and manganese.
3. Sulphide oxidation: If suphide minerals e.g. pyrite are exposed to oxygen, often at the water table, acidic (pH 1-6) and sulphate-rich waters are produced.
4. Geothermal waters: Waters of high temperature and arsenic correlated with chloride are produced from deep and sometimes volcanic waters that leach arsenic from the country rocks (Ravenscroft *et al*., 2009)

1.3.4.3 Anthropogenic arsenic contamination

Humans are responsible for arsenic pollution as a result of mining for coal and metal (Figure 1.15). The exposure and thus oxidisation of sulphide-rich ores to air and rainwater produces acid mine drainage which contains high levels of arsenic. Spoil heaps from mines can lead to contamination of soil that is present for decades after mining has ceased – for

example in the tin mines of Cornwall, UK (Ravenscroft *et al*., 2009). Additionally, the smelting of sulphide ores pollutes the air with arsenic and the burning of coal is a major contributor to arsenic in the surface environment. Pesticides previously contained high levels of arsenic and although this composition has been abandoned in Western countries arsenic pesticides are still in use in developing countries (Jayatilake *et al*., 2013)

1.3.5 Detection and assessment of exposure

1.3.5.1 Analytical techniques

Arsenic can be detected in water in the field using of field testing kits which utilise the reaction between zinc and water and arsenic to produce arsine gas (AsH_3) which reacts with mercury bromide paper and produces a colorimetric indication of the presence of arsenic (Williams *et al*., 2007a).

$$As(OH) + 3Zn + 6HCl \rightarrow AsH_3 + 3ZnCl_2 + 3H_2O$$

$$H_3AsO_4 + 4Zn + 8HCl \rightarrow AsH_3 + 4\ ZnCl_2 + 4H_2O$$

These tests are semi quantitative as the shade of colour on the mercury bromide paper relates to the quantity of arsenic gas produced. The test vary in their accuracy and to try and improve the results of field sampling UNICEF have now published a list of approved arsenic test kits[9].

Atomic absorption spectrometry (AAS) is a technique which quantitates arsenic through the absorption of optical radiation by gaseous free atoms (Chakraborti *et al*., 2003). ICP-MS ionises the arsenic atoms which are then detected by a mass spectrometer (Williams *et al*., 2007b). These analytical methods are reference methods which require

[9] www.unicef.org/supply/files/Monitoring_Arsenic_in_Water.pdf

skilled personnel and advanced laboratories[10] but are routinely used in research whilst the field kits are used for surveying and detection[9].

Both the field kits and AAS and ICP-MS detect "total" arsenic. If urine is being analysed it is important to know if a patient has consumed seafood in the preceding 4 days as the tests would detect arsenobetaine, a non-toxic organic form of arsenic that naturally occurs in fish (Vahter, 1994). If speciation is required then the reference methods can be coupled with high performance liquid chromatography (HPLC) (Engstrom *et al*., 2013).

1.3.5.2 Individual assessments of exposure

Arsenic exposure has been assessed in many ways. The concentration of arsenic in an individual's primary water supply together with daily volume consumption will give a good approximation, but, ideally, length of exposure should also be considered (Argos *et al*., 2010). This method does not take into account exposure from other tube wells, food and other sources. The latter's contribution can be considerable depending on the region (Ravenscroft *et al*., 2009). In view of this, arsenic in urine is a very valuable biomarker of the exposure over the last 24 h (Concha *et al*., 2006). Ideally this should be collected as a 24 h sample but many studies report spot urine for which there is not an established cut off. There is no definitive cut-off for elevated arsenic in urine. The normal range is thought to be 5-40 μg L^{-1} (Mandal and Suzuki, 2002; Ravenscroft *et al*., 2009). In 2001, D.N.G. Mazumder recommended a level of > 50 μg L^{-1} urinary arsenic (Mazumder, 2001), but this was to diagnose 'arsenicosis' and is coupled with drinking water at >50 μg L^{-1} and levels < 50 μg L^{-1} have been found to be relevant in disease pathogenesis. Urine arsenic levels can also be presented as μg g^{-1} creatinine but this is controversial as arsenic exposure itself can increase creatinine clearance (Gamble and Liu, 2005) (Basu *et al*., 2011). The arsenic level

[10] http://www.who.int/water_sanitation_health/dwq/chemicals/arsenic.pdf

in nails and hair gives an indication of exposure over the last 6 months; however removal of external contamination from water and soil is an issue and this measure correlates less well with primary water supply (Concha *et al*., 2006).

1.3.6 Health effects of arsenic

1.3.6.1 Exposure pathways

Arsenic can enter the body in 3 ways – respiratory, dermal and by ingestion. As mentioned in Section 1.3.5 respiratory exposure tends to be from industrial emissions or domestic fuel but can also be from aerial pesticide spraying, cigarette smoking and painted wallpaper (Ravenscroft *et al*., 2009). The dermal exposure pathway is through washing, bathing, swimming and working in arsenic contaminated water bodies (Lowney *et al*., 2005) as well as from non–aerial pesticide application, water effluents and phosphate detergents. Although inorganic arsenic can be readily absorbed in the gastrointestinal tract from food and water, dissolved arsenic is absorbed more easily. Therefore a weight per gram of food is less toxic than the same weight in water (Ravenscroft *et al*., 2009). However, in the rice eating communities of the Indian subcontinent the contribution of locally grown rice to arsenic exposure is not negligible (Williams *et al*., 2007b).

1.3.6.2 Acute arsenic poisoning

The lethal dose of arsenic for a human is 1-3 mg kg^{-1}. Acute poisoning causes 2 syndromes: acute paralytic syndrome where death occurs within hours secondary to cardiovascular collapse and depression of the central nervous system. More common is the acute gastrointestinal syndrome which is characterised by violent vomiting often leading to rupture of organs, diarrhoea, dehydration and circulatory failure. The progression to death

can be reversed by irrigation of the gastrointestinal system and chelation with dimercaprol (Kosnett, 2013).

1.3.6.3 Chronic arsenic poisoning

The levels of arsenic in well water has only rarely been reported to cause acute symptoms and lethality (Feinglass, 1973). However, in well-designed prospective longitudinal studies in Bangladesh arsenic exposure has been shown to increase all-cause mortality (Argos *et al*., 2010; Mogi *et al*., 1999). A trend of increasing mortality risk with increasing arsenic exposure was demonstrated which was applicable to the subgroups of cancer, infection and cardiovascular disease related deaths (Sohel *et al*., 2009).

1.3.6.3.1 Oncogenic effects

The association between arsenic exposure and mortality from cancers of the lung, liver, bladder, kidney and skin has well established since 1988 (Chen *et al*., 1988). The population of Antofagasta, Chile, demonstrates the delay in effect of arsenic exposure. For 12 years people were exposed to high levels of arsenic which was then removed (Yuan *et al*., 2007); 20 years later the cancer mortality rates rose. In Nepal, Vietnam, Myanmar and Cambodia with only a short duration of arsenic exposure it is thought that the rise in mortality rates is yet to come (Berg *et al*., 2007).

The mechanism of carcinogenicity has been extensively studied and include changes at a genetic level of aneuploidy, comutagenesis and delayed mutagenesis. Arsenic is thought to interact with poly(ADP ribsose) polymerase and to effect DNA repair through activation of p53 (Rossman and Klein, 2011). Other mechanisms of toxicity are oxidative stress, induction of apoptosis, chromosomal aberration, epigenetic modification, micronuclei formation and effects on stem cell populations (Dangleben *et al*., 2013).

Individuals who excrete a higher proportion of urinary MMA than DMA have a higher risk of skin cancers (Chen *et al*., 2003) indicating that MMA is a potent carcinogen.

1.3.6.3.2 Dermatological effects

The dermatological changes of hyperkeratosis, and hypo or hyperpigmentation, as an outward manifestation of arsenic exposure was established as early as 1984 (McDowell and Sacks, 1999). The dermatological changes typical of 'arsenicosis' (Section 1.3.6.3.4) include as well as palmar plantar hyperkeratosis and abnormal pigmentation of the skin, pigmentation involving the undersurface of the tongue and nodular lesions, verrucous lesions and cracks and fissures over palms and soles (Mazumder, 2001).

Bowen's disease, a precancerous lesion in the top layer of skin is often present. These skin changes can develop into the cancerous lesions of squamous cell carcinoma and leucomelanoma. There is a clear dose response between the level of arsenic in drinking water and the development of skin lesions as demonstrated in West Bengal in 1998 (Guha Mazumder *et al*., 1998). In Bangladesh people drinking arsenic contaminated water between 8.1-40 µg L^{-1} were 1.91 times as likely to have skin lesions than those drinking water <8.1 µg L^{-1}. This odds ratio (OR) rises to 5.39 for people drinking water between 175-864 µg L^{-1}. Based on a study by a Taiwanese group, The Agency For Toxic Substances and Disease Registry (ATSDR), developed a minimum risk level for development of dermatological lesions of 0.3 µg kg^{-1} day^{-1} (Tseng *et al*., 1968). It is thought that vascular, hepatic and neurological disease have similar thresholds.

1.3.6.3.3 Health effects on other systems

Arsenic exposure has been linked to many other diseases as well as cancer. The well-established associations are with peripheral vascular disease ('blackfoot disease' in Taiwan (Tseng *et al*., 1968) , cardio- and cerebro-vascular disease (Yuan *et al*., 2007), respiratory

Table 1.5 Diagnostic criteria for 'arsenicosis'

Adapted from (DNG Mazumder, 2001)

	Diagnostic category	Criteria
1.	**Exposure**	At least 6 months exposure to arsenic levels of greater than 50 µg L^{-1} or exposure to high arsenic levels from food or air
2.	**Dermatological**	Features of chronic arsenicosis: hyperkeratosis, hyper/hypopigmentation
3.	**Non carcinomatous manifestations**	Weakness, chronic lung disease, non cirrhotic portal fibrosis of liver with/without portal hypertension, peripheral neuropathy, peripheral vascular disease, non pitting oedema of feet/hand
4.	**Cancers**	Bowens disease, Squamous cell carcinoma, Basal cell carcinoma at multiple sites and unexposed regions
5.	**Biological evidence of exposure**	Arsenic level in hair/ nails > 1 mg kg^{-1} and 1.08 mg kg^{-1} respectively and/or arsenic level in urine > 50 µg L^{-1} (with a history of no recent seafood consumption)

A definite case is criteria 1-5, 1-4 or 2-5 if dermatological manifestations are moderate/severe.

A probable case is criteria 1-5 or 2-5 with mild dermatological manifestations, 2-4 with moderate/severe manifestations or 3 & 5 or 4 & 5.

illnesses including bronchiectasis, diabetes and peripheral neuropathy. Exposure in utero is thought to increase the risk of these diseases in adulthood (Smith *et al*., 2006). Increasing levels of arsenic exposure during pregnancy increases risks of infant mortality (Rahman *et al*., 2010). In addition arsenic has also been found to adversely affect the intellectual function of children and mental health in all ages (Tyler and Allan, 2014). Socially, the dermatological manifestations have considerable stigma and can prevent children from being sent to school and your women from being able to marry (Hassan *et al*., 2005).

1.3.6.3.4 Arsenicosis

Table 1.5 summarises the diagnostic criteria for the chronic disease "arsenicosis" (Mazumder, 2001). There is no satisfactory treatment although supplementation of diet with zinc, selenium and retinoic acid has been proposed. Discontinuation of the exposure is the most effective treatment (Ravenscroft *et al*., 2009) and can lead to improvement/regression of skin lesions in half of cases. However the cancer risk remains (Berg *et al*., 2007). Although chelation is effective in acute arsenic poisoning currently there is no indication for use of chelation therapy in chronic arsenic exposure[11] (Kosnett, 2013).

1.3.6.3.5 Geographical variation

There are geographical differences between the health effects of arsenic contamination (Ravenscroft *et al*., 2009). This may be related to the differences in water chemistry, genetic susceptibility of populations, methylation capacity and nutritional factors. The poor

[11] http://www.who.int/water_sanitation_health/dwq/arsenicun4.pdf

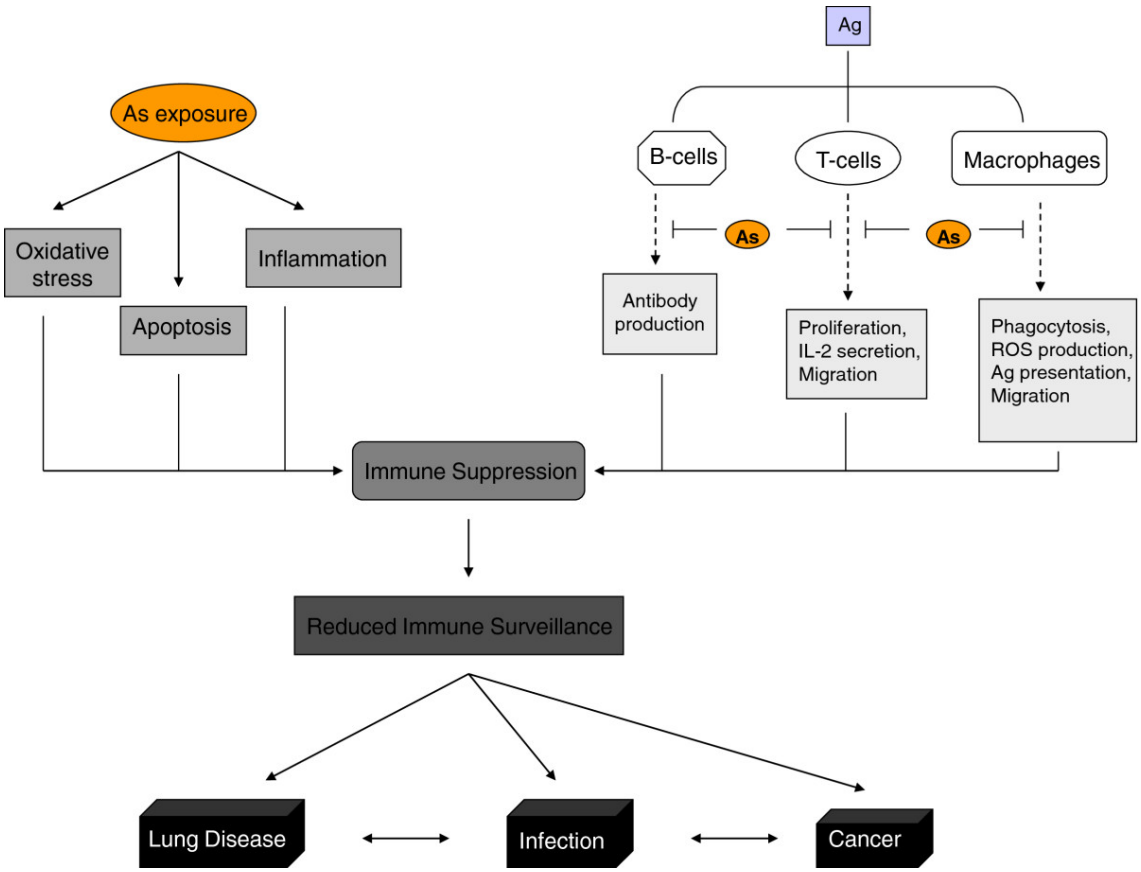


Figure 1.18 Summary of the effect of arsenic exposure on the immune system

Figure from (Dangleben *et al* 2013)

suffer the worst symptoms of arsenicosis[12] and nutritional deficiencies have been demonstrated to increase the risk of skin lesions (Mitra *et al*., 2004).

1.3.7 Arsenic and the immune system

Research on the relationship between arsenic and the immune system has largely been driven by the oncogenic effect of arsenic. However interest is growing on the interplay between arsenic and infectious diseases (Spivey, 2011). The studies of human immune cells extracted from arsenic-exposed subjects provide directly relevant data as there is controversy relating to the relevant dose conversion for *in vitro* and *in vivo* work and significant differences in arsenic metabolism in animal data. This is particularly relevant in immune studies due to arsenic's pleiotropic effect on the immune system at different doses. Figure 1.18 is a summary of the overarching effects of arsenic on the immune system drawing on data from *in vitro, in vivo* and clinical research (Dangleben *et al*., 2013). The results of human studies are outlined below.

Arsenic is progressively being shown to have immunotoxic effects (Dangleben *et al*., 2013). One study used microarrays of peripheral blood monocytes to compare 2 groups exposed to 0.7 μg L^{-1} and 32 μg L^{-1} in the US and demonstrated differences in T cell receptor signalling, increased expression of inhibitors of cytotoxic natural killer cell activity and decreased expression of defense response and inflammatory genes as well as MHC II DRB1, DQP1 and DPA1 in the exposed groups (Andrew *et al*., 2008).

Further evidence of decreased expression of inflammatory cytokines was found in studies from Bangladesh and Mexico (Argos *et al*., 2006; Salgado-Bustamante *et al*., 2010). These findings were contradicted by a study from Taiwan that found a generally pro-

[12] http://journals.lww.com/epidem/Fulltext/2005/09000/Social_Dimensions_of_Chronic_Arsenicosis_in_West.165.aspx#

inflammatory state with higher arsenic exposure; however it used blood levels as a marker of exposure and pooled samples (Wu *et al*., 2003). All these studies used small numbers and the contradiction in data may demonstrate responses to arsenic toxicity which change over time or differences in exposure assessment and study design.

Directly measured reductions in lymphoproliferation and cytokine levels of IFNγ, TNFα, IL-2, IL-4, IL-5, IL-10 were found in patients from West Bengal (Biswas *et al*., 2008). This contradicts a Chilean study of miners exposed to arsenic through inhalation (Escobar *et al*., 2010) where greater lymphocyte proliferation was seen in the arsenic exposed group.

Immunosuppression was confirmed further from studies on peripheral blood monocytes (PBMCs) from exposed individuals in Eastern India which were found to have inhibited macrophage function including reduced nitric oxide production and phagocytic function (Banerjee *et al*., 2009). Beta defensins, antimicrobial peptides associated with innate immune defense found in macrophages, granulocytes and NK cells, were also found to be inversely correlated urinary As levels in a population in Chile (Hegedus *et al*., 2008) and maybe particularly important in the development of the chronic lung disease bronchiectasis.

The effect of arsenic in children seems to follow a similar pattern. Arsenic exposed children in Mexico have lower levels of the major proinflammatory cytokine IL-2 and low $CD4^+$ cells together with elevated GM-CSF and decreased lymphocyte proliferation but no change seen in IL-4, IL-10, IFNγ, to PHA, $CD8^+$, B and NK cells (Soto-Pena *et al*., 2006). Arsenic exposure in different studies, has been shown to both increase and decrease levels of reactive oxygen species in children but a recent paper confirms a reduction in both the

Figure 1.19 Public health education picture, Samastipur, Bihar

This picture depicts a tube well tested for arsenic - blue painted: arsenic level < 50 µg L^{-1} red painted: ≥ 50 µg L^{-1}. The translation of the Hindi text is “Water fit for drinking and cooking/water not fit for drinking and cooking. P.H.D. Samastipur”.

P.H.D. = public health department. Photo: author’s own.

inflammatory cytokines Tumour necrosis factor-α (TNF-α) and IL-2 (Ahmed *et al*., 2014). The exposure of the foetus to arsenic, as it readily crossed the placenta, has an important effect on the infant immune system as it is associated with an increased risk of lower respiratory tract infections (69%) and diarrhoea by (20%) in infancy (Rahman *et al*., 2011).

There are few direct studies of arsenic's effect on outcome of infectious diseases in humans although the above studies point towards a compromised response to pathogens. A Chilean study reported an increased risk of tuberculosis 20 years post high level exposure (Smith *et al*., 2011). Animal studies have reported delayed clearance of Influenza A (Kozul *et al*., 2009) and *Staphylococcus aureus* (Bishayi and Sengupta, 2003) but a faster clearance of *Giardia muris* (Escudero-Lourdes *et al*., 2005). These contradictory results may be related to dose or the type of organism. Further work is required.

1.3.8 Mitigation of arsenic exposure

There is not a simple or easy strategy for arsenic mitigation of a water supply for a community. There are 3 main approaches that have been employed: treatment of the contaminated water, development of a surface water supply or sinking of an alternative well either deeper or in another location. Educating the users of a well by testing the water and painting the result on the well: red for unsafe and blue for safe (Figure 1.19), is the most effective method so far with a third of villagers switching their wells (Ahmed *et al*., 2006).

1.3.8.1 Treatment of contaminated water

The methods of arsenic removal are many and varied and tend to work better for As^{V}. They include oxidation and filtration using membranes or sand, lime softening, adsorption onto metal oxides, ion exchange resins and phytofiltration (Ravenscroft *et al*., 2009). These systems require community acceptance, safe removal of toxic waste and regular

maintenance and funding. These are prevalent issues in many villages in Bihar leading to defunct systems as discovered by a recent survey conducted by 2 Dutch students for their Masters' degree from Delft University (Brouns *et al*., 2013).

It is also possible to try and treat the aquifer itself by cyclically injecting oxidised water or compressed air into reducing aquifers (Brunsting and McBean, 2014). Another option is performing an aquifer 'clean-up' where the finite amount of arsenic is extracted from the aquifer. This latter technique risks drawing down arsenic from other sources (Ravenscroft *et al*., 2009).

1.3.8.2 Use of surface water

The use of treated surface water and the return to conventional dug wells has been strongly supported in Bangladesh's National Policy for Arsenic Mitigation[13] and by arsenic trace analytical specialists (personal communication Dipankar Chakraborti and Joerg Feldmann). Unfortunately, the control of human pathogenic bacteria remains a serious issue and contamination has still been found in the use of modified traditional dug wells despite attempts to control this (Hira-Smith *et al*., 2007).

1.3.8.3 Accessing groundwater at a deeper level

The digging of deeper wells is positively viewed and has the advantage of low maintenance and no waste production (Ahmed *et al*., 2006). In forty six wells >150 m deep, from across the arsenic-polluted area of south-central Bangladesh, groundwater composition remained unchanged between 1998 and 2011 (Ravenscroft *et al*., 2013). The disadvantage of this approach has the unknown factor of what risks extensive use of deeper aquifers will bring.

[13] http://www.dphe.gov.bd/pdf/National-Policy-for-Arsenic-Mitigation-2004.pdf

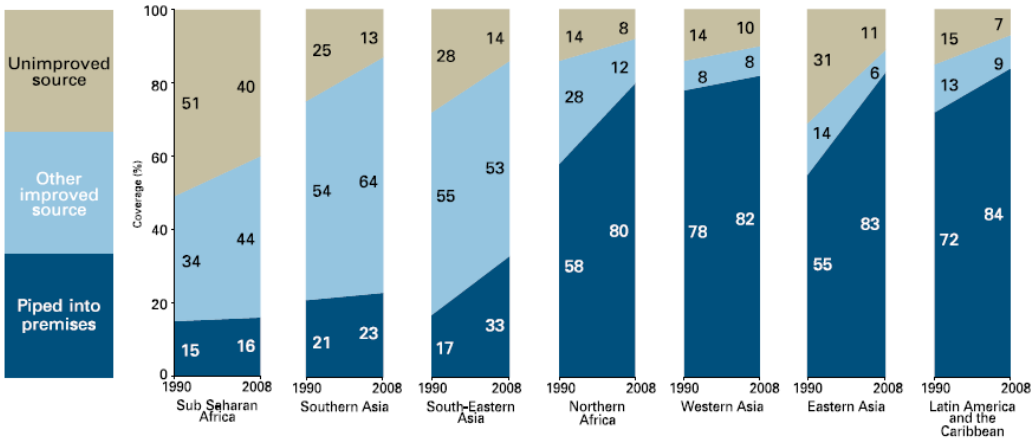


Figure 1.20 Distribution of water sources in low – income countries

Source: Drinking water. Equity, safety and sustainability. UNICEF, WHO 2011.

1.3.8.4 Support for arsenic mitigation

Multiple non-governmental organisations are involved in arsenic mitigation with the disadvantage of uncoordinated activity and inconsistent cover and funding as alluded to in Bihar in Section 1.3.8. Consequently, UNICEF and the WHO have taken the approach of supporting the public health and education departments in affected countries in health and sanitation education and installation of appropriate community treatment plants and other mitigation strategies[14]. The Indian Bureau of National Standards still has a limit[15] of 50 µg L^{-1} arsenic but is receiving pressure from UNICEF and WHO for this to be reduced in line with the WHO limit of 10 µg L^{-1}. The ultimate solution of piped water for everyone is part of their long term goal though would require enormous resources for installation (Figure 1.20) and maintenance in these rural areas.

1.3.9 Resistance to arsenic in microorganisms

Resistance selection of microorganisms to environmental arsenic has been well studied. From an evolutionary perspective As^{III} transporters were present in micro-organisms first due to exposure to As^{III} and the arsenic reductases appeared when the environment became oxidising 3.8 billion years ago well before eukaryotes 850 Mya. This is a good 400 million years before the kinetoplastids. The mechanism of arsenic resistance developed was through the ARS operon which exists in 3 parts, ArsC, an arsenate reductase produces As^{III} which reacts with ArsB to activate ArsA which is a soluble ATPase subunit that effluxes As^{III} from the cell (Mukhopadhyay *et al*., 2002). Loss of the ArsC leads to sensitivity to arsenate in *Escherichia Coli* (Mukhopadhyay *et al*., 2002). The only identified eukaryotic arsenic reductase is the yeast Arc2p which is the enzyme which led to the identification of

[14] JMP Thematic Report on Drinking Water 2011
http://www.wssinfo.org/fileadmin/user_upload/resources/report_wash_low.pdf
[15] http://bis.org.in/sf/fad/FAD25(2047)C.pdf

Figure 1.21 District distribution of arsenic contamination, VL endemicity and antimonial resistance in Bihar

Figure from (Perry *et al* 2011).

LmACR2 (Zhou *et al*., 2004). It is paradoxical that the function of reductases that evolved to protect against arsenate, which has its own unique toxicity in interference with cellular respiration as a phosphate analogue, maybe responsible for the activation of Sb^{V} to the toxic Sb^{III} and hypothetically without which cells are resistant to Sb^{V}.

1.4 Hypothesis

This study aims to explore a hypothesis that draws together arsenic, antimony and *Leishmania.* We propose that arsenic contamination of drinking water may have contributed to antimonial treatment failure.

The Indian sub-continent is the only place world-wide where arsenic contamination and VL co-exist and it is the area where the highest rates of antimonial treatment failure are found. Of note, antimonial contamination of the ground water is not thought to be an issue in this region (McCarty *et al*., 2004). In the map of Bihar in Figure 1.21 information on VL endemicity, antimonial treatment failure and arsenic contamination has been collated. Even in the context of under-reporting of VL and its treatment outcomes and the absence of a state-wide arsenic survey there are 10 districts where antimonial treatment failure in VL and arsenic contamination co-exist (Perry *et al*., 2011). There is minimal reported arsenic contamination in the north of Bihar yet resistance is present at high levels (Sundar *et al*., 2000). Resistant strains may have disseminated through their stability and established increased fitness compared to sensitive strains. Worker migration (Deshingkar *et al*., 2006) is also common between the south and the north. In Bangladesh where minimal antimonial resistance has been reported, arsenic contamination is found in the south and VL in the north.

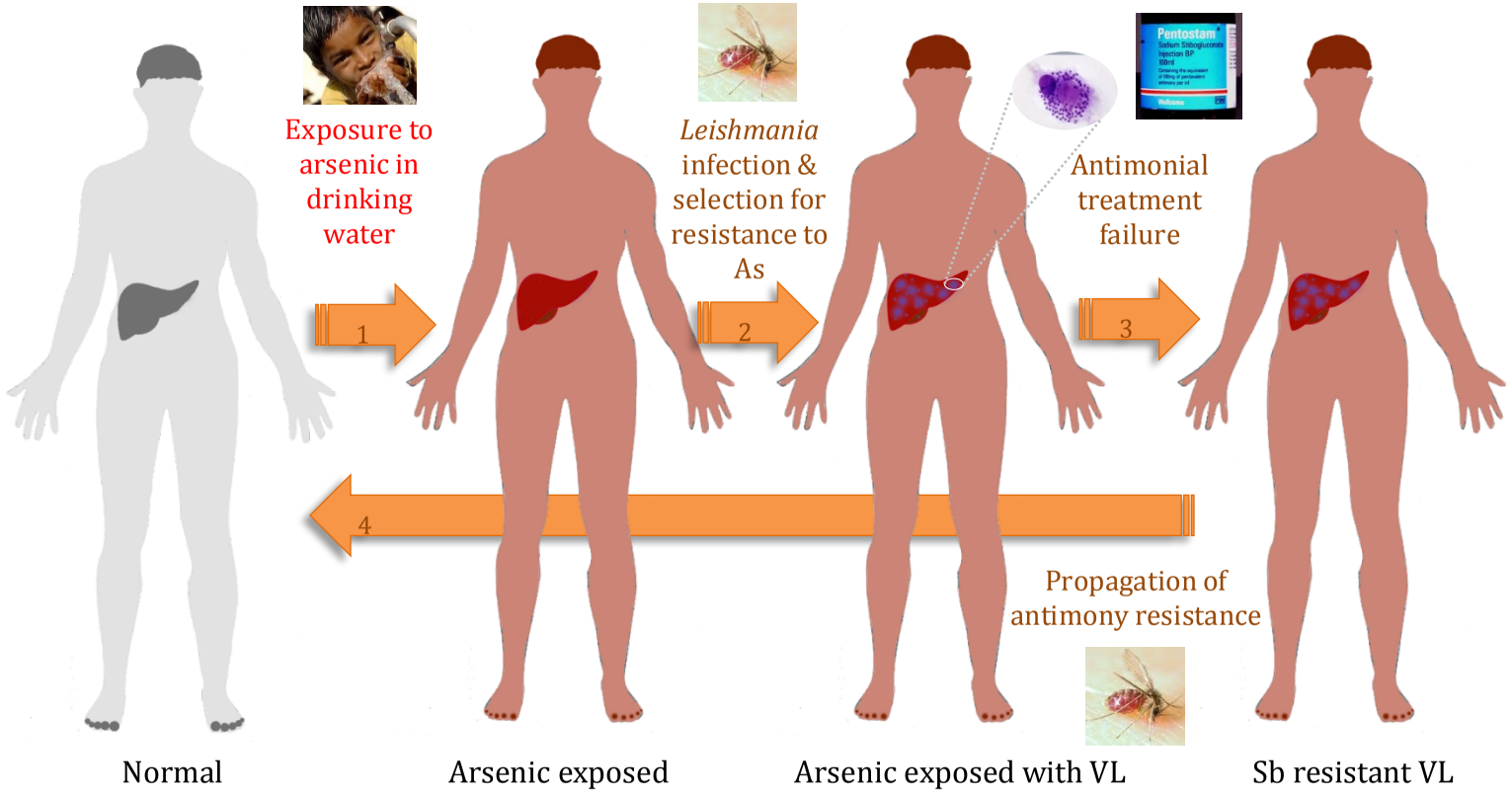


Figure 1.22 Arsenic hypothesis of antimony resistance in visceral leishmaniasis

Step 1: drinking of arsenic contaminated water leads to an accumulation of arsenic in skin, hair, nails and liver. Step 2: upon infection with *Leishmania* the parasite is exposed to arsenic within the liver and develops resistance mechanisms (Section 1.2.4). Step 3: these resistance mechanisms are then effective against antimonial treatment when administered. Step 4: antimonial resistant parasites have a fitness advantage and are preferentially transmitted leading to a propagation of resistance throughout the population (Vanaerschot *et al*, 2010) and (Vanaerschot *et al*, 2011)

As noted in Section 1.2.5 exposure of *Leishmania* parasites to arsenic *in vitro* leads to cross resistance to antimonial species. The metabolism of arsenic within the human body (Section 1.3.2) is such that there is accumulation in the liver over time at levels up to 6 mg kg^{-1} dry weight (Mazumder, 2005). Levels of arsenic in the other organs that *Leishmania* resides in (spleen and bone marrow) have not been established in humans though high splenic accumulation has been described in the offspring of orally exposed Wistar rats (Zhang *et al*., 1998).

If arsenic was freely available in the liver and other organs it is possible that high levels of arsenic exposure could be partially protective against established infection with *Leishmania*. What is more likely is that the exposure to arsenic that the *Leishmania* parasite would experience within a chronically arsenic exposed person is sub-lethal. Although accumulation of arsenic within macrophages has not been directly established it can be assumed that this occurs as antimony accumulates well in macrophages and both As^{III} and Sb^{III} are taken up by AQP9 in undifferentiated human leukaemia cells (Bhattacharjee *et al*., 2004).

Our hypothesis is that arsenic exposure would allow the parasite to develop mechanisms of resistance over the clinical incubation period of weeks to months. These mechanisms of resistance would be effective against antimonial treatment leading to treatment failure (Figure 1.22).

Development of arsenical resistance through sub-acute exposure is a process that is well researched in micro-organisms (Mukhopadhyay *et al*., 2002). This hypothesis also has parallels with the administration of chloroquine-treated salt to the populations of South America, South East Asia and Africa that is thought to have contributed to the development of chloroquine resistance (Payne, 1988).

It is worth noting that *Leishmania*, antimonial treatment and arsenic exposure have significant effects on the immune system (Section 1.1.5, 1.2.4.4, 1.3.7). Perturbation of immune function, as well as biochemical causes, may also contribute to treatment failure of VL with antimonial compounds in arsenic exposed areas.

1.5 Aims of project

This overall of this project is to investigate the relationship between arsenic, antimony and *Leishmania*.

The specific aims are to:

- Investigate the development of resistance in *Leishmania* to antimonials through different methods of exposure *in vitro* to the metalloids arsenic and antimony
- Develop an environmentally relevant model of arsenic exposure in BALB/c mice
- Establish proof of concept in the laboratory of the hypothesis that arsenic contamination of drinking water could induce antimonial resistance in *Leishmania* using an *in vivo* model
- Evaluate, through a field study, whether arsenic exposure is related to antimonial treatment failure in Bihar, India.

Chapter 2 -
Materials and methods

Section A: laboratory work

2.1 Material

All chemicals were from Sigma Aldrich unless otherwise stated. The highest grade and purity of reagents and chemicals that are available were used in this work. The solids of Sb^{V} as sodium stibogluconate, free of *m*-chlorocresol was a kind gift from GlaxoSmithKline, Sb^{III} was used as potassium antimony tartrate and As^{III} as sodium meta arsenite. Concentrations of antimony compounds are traditionally quoted in μg ml^{-1} using the absolute amount of Sb content within the compound. However throughout, for ease of presentation with arsenic and other compounds, results will be presented in molar form with μg ml^{-1} in brackets. MMA^{III} and DMA^{III} were synthesised by Dr. Andrea Raab at Aberdeen University.

2.2 Animals and animal ethics

Golden Syrian Hamsters, BALB/c and C3H mice were commercially acquired from Harlan. All animal experiments were performed in compliance with the Animals (Scientific procedures) Act 1986 (UK Home Office Project License PPL 60/4039) in accordance with the European Communities Council Directive (86/609/EEC) and approved by the Ethical Review Committee at the University of Dundee.

Table 2.1 Composition of different media

Media	Cell type	FBS*, %	Composition
LdBob M199 media	promastigote	10	M199 (Caisson Labs) 25 mM HEPES 12 mM $NaHCO_3$ 1 mM glutamine/1x GlutaMAX 1x RPMI 1640 vitamin mix 10 μM folic acid 100 μM adenosine 150 μg ml^{-1} haemin
LdBob axenic media	amastigote	20	15 mM KCl 136 mM KH_2PO_4 10 mM $K_2HPO_4.3H_20$ 0.5mM $MgSO_4.7H_20$ 24 mM $NaHCO_3$ 22 mM glucose 1 mM glutamine/1x GlutaMAX 1 x RPMI 1640 vitamin mix 10 μM folic acid 100 μM adenosine 1x RPMI amino acid mix 5 μg ml^{-1} haemin 0.0005% phenol red 25 mM MES
M199 media	promastigote	10 %	M199 (Caisson Labs) 4 mM HEPES 100 μM adenosine 50 μg ml^{-1} haemin
Grace's Media	promastigote	10%	200 μg ml^{-1} haemin
Supplemented RPMI	promastigote	20 %	RPMI 1640 (R0883) 20 mM HEPES 100 μM adenine 3.26 μg ml^{-1} haemin 3 μM dihydrobiopterin 1 % penicillin/streptomycin 1 mM glutamine/1x GlutaMAX
DMEM	*ex vivo* amastigote	10 %	Dulbecco's Modified Eagle's Medium (Bio Whittaker)
RPMI	macrophage	20%	RPMI 1640 (R0883)

* Foetal Bovine Serum (GE Healthcare)

All media adjusted to neutral pH except LdBob axenic amastigote media pH 5.66 at 22°C (pH 5.5 at 37°C).

2.3 *Leishmania* cell culture

Media used for culturing cells was filter-sterilised using filtering units with a 0.22 μm PES (polyethersulfone) membrane (Nalgene or Steriflip). All *Leishmania* cultures were routinely grown, with or without shaking at 200 rpm, over 72 h to a density of 2 -3 x 10^7 ml^{-1} which represented stationary phase and then passaged into fresh medium at a density of 5 x 10^5 ml^{-1}.

2.3.1 *L. donovani* 1S2D (LdBob)

A clonal line of *L. donovani* 1S2D (WHO designation: MHOM/SD/62/1S-CL2D), also known as LdBob (Goyard *et al*., 2003), was used in this study. LdBob has the ability to differentiate readily between promastigotes and axenic amastigotes. In the promastigote stage parasites were grown in LdBob M199 medium (Table 2.1) and incubated at 28 °C.

For differentiation to axenic amastigotes, stationary phase promastigotes were passaged into LdBob amastigote medium (Table 2.1) at pH 5.5 and grown at 37 °C with 5% CO_2. Parasites were maintained for at least 2 passages in the appropriate medium prior to commencement of any experiment. For routine culture, parasites were cycled between developmental stages every 4-5 passages.

2.3.2 LV9

L. donovani promastigotes LV9 strain (WHO designation MHOM/ET/67/HU3) were cultured by serial passage in supplemented Grace's Insect Medium (BioWhittaker) at 28 °C. Amastigotes of *L. donovani* LV9 were freshly isolated from the spleen of infected Golden hamsters, as previously described (Wyllie and Fairlamb, 2006b). Briefly, infected

spleen were homogenised and washed in Dulbecco's Modified Eagle's Medium (BioWhittaker) supplemented with 10% FCS (v/v) (2600 g, RT, 6 min) (Glaser *et al*., 1990). Red cells were lysed using saponin 0.1% (w/v) and 2 further washes performed prior to passage through a 23G needle and numeration as in section 2.3.3. These *ex vivo* amastigotes were used directly for experimentation or serially passaged through BALB/c mice by intraperitoneal (i.p.) injection of 0.2 ml of 1×10^7 amastigotes harvested from freshly prepared splenic homogenate every 1-2 months.

For differentiation of *ex vivo* amastigotes to promastigotes, an aliquot of the *ex vivo* amastigote preparation was placed in either supplemented Grace's Insect Medium as above or in 20 % FCS RPMI media (Table 2.1).

2.3.3 Cell density measurements

Leishmania were fixed in 2 % formaldehyde with a dilution of 1 in 10 prior to being counted manually using a Neubauer haemocytometer chamber under a light microscope (Zeiss). Alternatively, cell size and number were determined using a CASY® cell counter according to manufacturer's guidelines (Model TT Sharfe). Macrophages were enumerated using the same methods only without fixing and dilution.

2.3.4 Cloning

Parasites were cloned by limiting dilution. Following determination of cell density, the cell suspension of the relevant line was diluted to 1.5 cells ml^{-1} and dispersed as 200 µl aliquots in 96 well plates to obtain the equivalent of one parasite every 3 wells. Following a 7 -10 days incubation viable parasites were available for transfer to culture flasks in up to a third of the wells.

2.4 Macrophage cell culture

THP-1 cells (ECACC) were grown in RPMI-1640 medium (Invitrogen) (Table 2.1) and maintained between the concentrations of 3-8 (x 10^5) cells ml^{-1} at 37°C with 5% CO_2. Differentiation of THP-1 monocytes to macrophages was achieved by adding 10 nM phorbol 12-myristate 13-acetate (PMA) (Park *et al*., 2007) to cultures for 24-48 h.

For mouse derived macrophages BALB/c mice were prestimulated with an intraperitoneal injection of 2% soluble starch (w/v). Peritoneal macrophages were harvested at least 24 h later using peritoneal lavage with ice cold phosphate buffered saline with 5 mM EDTA. Harvested cells were then centrifuged at 350 x g for 10 min at 4 °C and the resulting pellet was incubated with 1 ml of Red Cell Lysis buffer (Sigma, 8.3 g L^{-1} ammonium chloride in 0.01 M Tris-HCl buffer, pH 7.4) for 1-2 min. This solution was diluted with RPMI macrophage medium (Table 2.1) and two further washes with RPMI at (350g, 5 min, 4 °C) 2 were carried out.

2.5 Growth inhibition studies (EC_{50})

The EC_{50} of a compound is the effective concentration at which cell growth is inhibited by 50%. In 96 well plates (TPP/Greiner) fresh media, containing varying concentrations of the compound to be tested, were incubated with *Leishmania*, from a stock culture of cells at log phase (1 x 10^7 cells ml^{-1}), at a final concentration of 1 x 10^5 cell ml^{-1}, as previously described (Jones *et al*., 2010; Shimony and Jaffe, 2008). The cells were incubated in the presence of the drug for 72 h and following a 3 h incubation with resazurin at a final concentration of 5 μM, the plates were read for the formation of resorufin, using a fluorescent microplate reader at $\lambda_{excitation}$ = 528 nm and $\lambda_{emission}$ = 590 nm.

Data were corrected for background fluorescence and expressed as a percentage of non-

treated cells. EC_{50} values were determined using the EC_{50} 3 parameter equation provided with the computer programme GraFit:

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}} \right)^{s}}$$

where y is the percentage growth at inhibitor concentration '*x*' and '*s*' is the slope factor. EC_{10} can be calculated from the same equation. Measurements were repeated in triplicate (unless otherwise stated) and data presented as weighted mean ± weighted standard error of the mean where 'a', 'b' and 'c' are the errors of the mean values of 'A', 'B', 'C'….'H' respectively.

$$= \frac{\frac{A}{a^2} + \frac{B}{b^2} + \frac{C}{c^2} + \ldots + \frac{H}{h^2}}{\frac{1}{a^2} + \frac{1}{b^2} + \frac{1}{c^2} + \ldots + \frac{1}{h^2}}$$

If parasite growth in a 96 well plate was sub optimal then the cells were incubated with drug in 25 cm^2 culture flasks (Cell star) for 72 h and 200 µl aliquots incubated in triplicate in a 96 well plate with resazurin as above. The same method was used for the determination of macrophage EC_{50} values using a starting concentration of macrophages of 2.5 x 10^5.

2.6 Selection of drug-resistant axenic amastigote cell lines

Wild type LdBob axenic amastigotes were cloned by limiting dilution (Section 2.3.3). Four of the resulting clones were tested for their sensitivity to trivalent arsenic (As^{III}) and antimony (Sb^{III}) and LdBobSu3 was chosen as the starter clone for the selection of resistant lines. Four lines were cultured in parallel with continuous exposure to As^{III}, Sb^{III}, Sb^{V} and

no drug, respectively. The cells were continuously passaged as axenic amastigotes throughout drug selection. Starting at a sublethal concentration of the relevant compound, approximately equal to the established EC_{10}, the drug concentration in the culture medium was increased in a stepwise fashion, as previously described (Detke *et al*., 1989; Dey *et al*., 1996). At regular intervals, cells were cryopreserved prior to the next increase in drug concentration. Parasites were cultured until they were able to grow at 64 times the starting concentrations of As^{III} and Sb^{III} and 32 times the starting concentration of Sb^{V}. These lines were then cloned in the absence of drug and tested for susceptibility to both metalloids in axenic amastigote form and intracellular form.

2.7 Optimisation of in macrophage assay

The following conditions were examined to optimise an assay for determination of the EC_{50} of amastigotes in their intra-macrophage environment (Chang and Dwyer, 1978):

- Parasite/macrophage ratios – 40:1, 10:1, 2:1
- Macrophage cell line – differentiated THP-1, mouse peritoneal macrophages
- Parasite cell line – promastigote, axenic amastigote, *ex vivo* amastigote
- Infection incubation time
- Medium and serum proportion 10 vs 20%
- Length of time course

In the final assay harvested mouse peritoneal macrophages (Section 2.4) were plated in Lab-Tek glass 8 well chamber slides (VWR International, Thermo Fisher Scientific) at a concentration of 1×10^5 cells per well. Cells were left to adhere in RPMI 20% medium for at least 2 h prior to infection with 1×10^6 cells per well of freshly harvested *Leishmania ex*

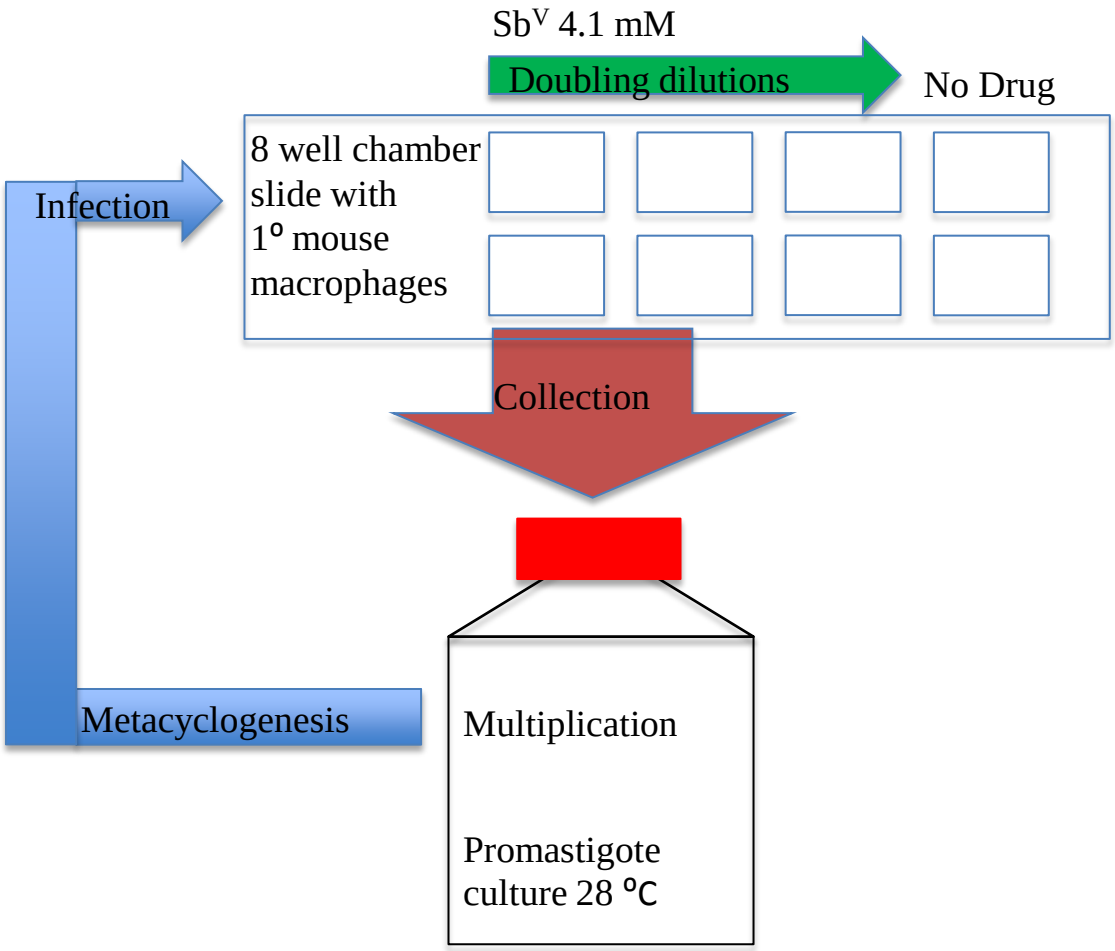


Figure 2.1 Generation of antimonial resistant *Leishmania* in macrophage

Starch elicited macrophages harvested from mouse peritoneum were plated and infected with late stationary phase promastigotes. Following infection the doubling dilutions of SbV were added with a top concentration of 4.1 mM. Following a 5 day incubation the medium was replaced with supplemented RPMI promastigote medium, the infected macrophages were mechanically disrupted and the amastigotes allowed to differentiate back into promastigotes for 7 days at 28 ^{0}C. Promastigotes grown from the well with the highest SbV concentration were harvested and expanded in culture bottles. On reaching late stationary phase the cycle was repeated. Following three cycles EC50 values were determined to antimonial compounds in both promastigote and intracellular amastigote form. Method and figure adapted from (Hendrickx *et al.*, 2012).

vivo amastigotes or late stationary phase promastigotes from *in vitro* culture. Following a 4-h incubation, the wells were washed with Hanks' balanced salt solution (Gibco) to remove any remaining non-phagocytosed parasites. Infected macrophages were incubated for 72 h with varying concentrations of drug in RPMI 20 %. The slides were then fixed in ice-cold methanol and stained with Giemsa (BDH, VWR International) for 30 min. The number of infected cells and number of amastigotes per cell were determined in 100 macrophages using a light microscope (Zeiss Standard 20) with a 100x oil immersion lens. EC_{50} values were determined as described in section 2.5.

2.8 Selection of resistance *in macrophage*

LV9 promastigotes were cloned by limiting dilution (Section 2.3.4). One of the resulting clones was split into 6 parasite lines, 3 of which were exposed as intracellular amastigotes to Sb^V up to 500 μg ml^{-1} in macrophage for 5 day periods interspersed with non-exposed promastigote cycles (Figure 2.1)(Hendrickx *et al*., 2012). The other 3 parasite lines were cycled through macrophages in the absence of drug. Differentiation from amastigote to promastigote form was achieved by changing to promastigote RPMI medium, mechanical disruption of the infected macrophage culture and incubation at 28 °C. After 3 amastigote/promastigote cycles EC_{50} values were obtained for both promastigote and intracellular amastigote forms.

2.9 Arsenic and antimony detection and quantification

2.9.1 Indicators

The following indicators were purchased from Invitrogen and evaluated for their ability to detect and differentiate between Sb^{III} and Sb^V:

- Newport green (2 μM)
- Phen Green (2 μM)
- TSQ (*N*-(6-methoxy-8-quinolyl)-*p*-toluenesulfonamide) (10 μM)
- Calcein (0.5 μM)
- Calcium Green (0.2 μM)

Indicators were diluted to their recommended concentrations (in brackets above) and incubated with 10-fold concentrations, ranging from 1 mM to 1 μM, of Sb^{III}, Sb^{V} or a control compound containing Zn^{II} ($ZnCl_2$). The control was TPEN. The plates were read on an EnVision plate reader at $\lambda_{excitation}$ = 473 nm and $\lambda_{emission}$ = 520 nm.

2.9.2 Atomic absorption spectrometry

This method was used in the analysis of arsenic levels in samples acquired during field work studies from India and a full description is found in the field work methods, Section 2.18.

2.9.3 Inductively coupled Mass Spectrometry

All arsenic measurements were performed in collaboration with Professor Joerg Feldmann's group at the University of Aberdeen. Final sample preparation was performed in Aberdeen by me or Dr. Andrea Raab and analysis was performed by Dr. Andrea Raab.

Samples (100-mg wet weight where possible) were weighed out into polypropylene tubes and 0.5 ml nitric acid was added and left to incubate overnight. One hundred microliters of H_2O_2 was then added and the samples were digested using microwave heating (temperature program: to 55 °C for 10 min, 75 °C for 10 min then 90 °C for 30 min) (Williams *et al*., 2007b). The samples were then diluted to 5 ml with double distilled water. For water samples, no digestion or dilution was performed.

An Agilent 7500c (Agilent Technologies, Tokyo, Japan) ICP–MS with a Meinhard nebulizer was used to measure the elements As (*m/z* 75) and Sb (*m/z 121*). Each sample was measured in triplicate and expressed as mg (kg wet weight)$^{-1}$. The average limit of detection was 0.2 μg As (kg wet weight)$^{-1}$. Arsenic values measured in certified reference material (Seronorm Trace Elements Urine; Seronorm) were 197 ± 5 μg L^{-1} of As (n = 3, certified value 184 ± 17 μg L^{-1} of As), and those measured in TORT-2[16] were 20 ± 0.4 mg (kg dry weight)$^{-1}$ of As (certified 21.6 ± 1.8 mg (kg dry weight)$^{-1}$of As.

To determine the conversion factor from wet weight to dry weight, livers and spleens of 3 BALB/c mice were harvested, weighed and then freeze dried (Micro Modulyo, Thermo Savant) for 48 h. The dried organs were then weighed and the ratio of wet to dry weight was calculated with standard deviation.

2.10 Arsenic exposure in BALB/c mice

An arsenic exposure model was designed to recreate two environmentally relevant situations reported from the Indian sub-continent within our mouse model. An established dose-equivalent equation based on body surface area was used to equate arsenic exposure in humans to mice (Reagan-Shaw *et al*., 2008):

$$\text{human equivalent dose (mg kg}^{-1}) = \text{animal dose (mg kg}^{-1}) \times (\text{animal } K_m/\text{human } K_m)$$

where K_m is weight/body surface area of the relevant mammal (mouse K_m = 3, human adult K_m = 37). A 60-kg man drinking 3 L day^{-1} (Watanabe *et al*., 2004) of water contaminated

[16] Lobster Hepatopancreas; National Research Council Canada; http://archive. nrc-cnrc.gc.ca/obj/inms-ienm/doc/crm-mrc/eng/TORT-2_e.pdf)

with arsenic at 1,200 μg L^{-1} is equivalent to the intake of BALB/c mice drinking water containing As^{III} at 10,000 μg L^{-1} (hereafter 10 ppm is equivalent to 133 μM). This concentration has been reported in arsenic-contaminated tube wells in Bihar (Chakraborti *et al*., 2003). Levels of up to 6 mg kg^{-1} (dry weight) have been reported from liver biopsies in patients exposed to 220 to 2000 μg L^{-1} arsenic for 1 to 20 years (Mazumder, 2005). Although the higher level of 100,000 μg L^{-1} (hereafter 100 ppm, equivalent to 1.33 mM) is equivalent to concentrations greater than those reported from Bihar this concentration was chosen to recreate these elevated hepatic arsenic levels over months rather than years.

Six to ten week old BALB/c mice were divided into three separate groups that received either normal tap water (hereafter 0 ppm), As^{III} at 10 ppm (10 mg L^{-1}), or As^{III} at 100 ppm (100 mg L^{-1}). Fresh water was given to all groups once or twice a week, water intake was measured, and the weights of the animals were measured weekly. All mice were fed a standard autoclavable murine chow pellets (RM1 A, Special Diets Services).

Three mice from each exposure level were euthanized at days 7, 14, 28 and 56. Harvested liver and kidney were placed in pre-weighed CK14 tubes containing ceramic beads with 500 μl double-distilled water. Harvested spleen was placed in pre-weighed MK28R tubes containing metal beads with 500 μl double-distilled water. Both types of tubes were supplied by PeqlabBiotechnologie GmbH. The samples were homogenized at 2 × 5,000 rpm for 10 s with a 20 s gap, using a PreCelleys 24 homogenizer (Bertin Technologies). Harvested bone was crushed with a pestle and mortar before mechanical homogenization, in pre-weighed MK28R tubes with 500 μl double-distilled water, at 2 × 5,900 rpm for 30 s with a 15 s gap. Separation of blood cells and sera was achieved by centrifugation at 15,000 × g for 90 s. Murine chow was also included for digestion and analysis.

2.11 Arsenic and macrophages

Peritoneal macrophages from mice exposed to arsenic at 0, 10 and 100 ppm for 28 or 56 days were harvested following pre-stimulation with 2% (w/v) starch as in section 2.4. The sensitivity of the resulting macrophages to As^{III} and Sb^{III} was determined using the resazurin assay (Section 2.5). The exposed macrophages were also assessed for their ability to kill intracellular amastigotes in the in-macrophage assay described above. Further exposed macrophages were pelleted and freeze dried and analysed for arsenic content with ICP-MS as described in 2.8.3.

Peritoneal macrophages from BALB/c mice and differentiated THP-1 cells were incubated with 0, 1, 2, 4 and 8 μM As^{III} for 72 h. They were then removed by treatment with lidocaine HCl (4 mg ml^{-1}) plus 5 mM EDTA using a cell scraper, then pelleted by centrifugation, snap frozen and freeze dried (Micro Modulyo, Thermo Savant) for 24 h before preparation for ICP-MS, as above.

2.12 *In vivo* selection of Sb^{V} resistant line

Six to ten week old BALB/c mice were exposed to As^{III} at 0, 10, or 100 ppm in their drinking water for 1 month prior to infection by i.p. injection of 0.2 ml of 1×10^7 *L. donovani ex vivo* splenic amastigotes from BALB/c mice, as described (Section 2.3.2). At 28 days post-infection, the mice were euthanized, hepatic and splenic imprints taken, fixed in methanol, stained with Giemsa and parasites quantified, as described below. The spleens of each group were prepared separately for harvest of *ex vivo* amastigotes. These amastigotes were used in the in-macrophage assay (Section 2.7), for assessment of their

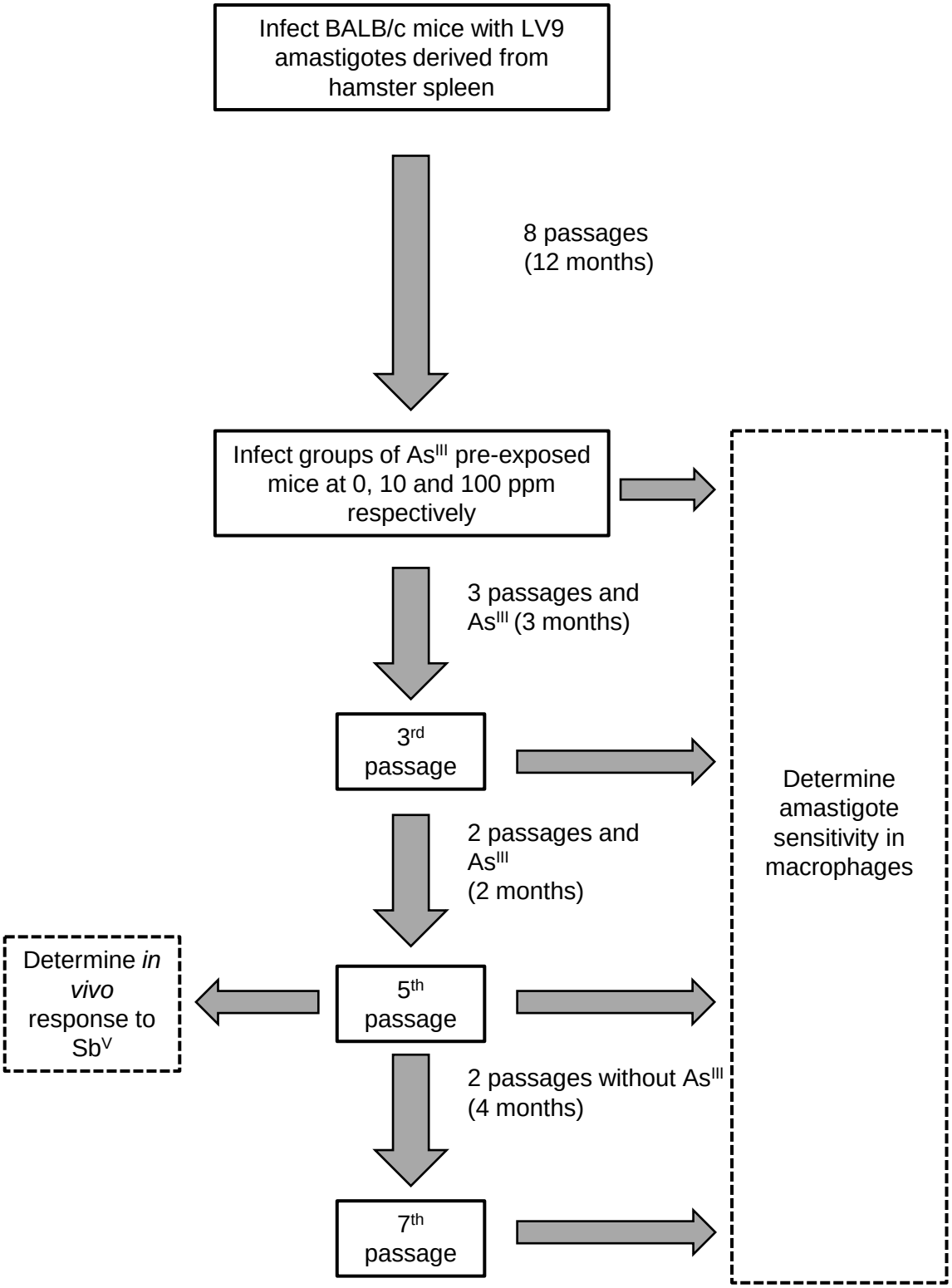


Figure 2.2 Experimental flow diagram of generation of antimonial resistant *Leishmania in vivo* through oral exposure to As^{III}

sensitivity to Sb^{V} and to reinfect further arsenic pre-exposed groups of mice. See Figure 2.2 for an experimental flow diagram. *Leishmania*-infected liver and spleen were also prepared and analyzed for arsenic content by ICP-MS, as above. After the fifth passage, *ex vivo* amastigotes were used to infect groups of non-arsenic exposed mice to assess stability of resistance. Following 4 months of passaging in BALB/c mice, these parasites were reassessed for their sensitivity to Sb^{V} *in vitro*.

2.13 *In vivo* assessment of Sb^{V} resistance

At the end of the fifth passage *in vivo* sensitivity to Sb^{V} and miltefosine was assessed: at 28 days post-infection, groups of mice from each exposure level were treated with Sb^{V} (50 mg kg^{-1}, subcutaneous (s.c.)), miltefosine (12 mg kg^{-1}, orally) or with drug vehicle only (sterile water, s.c. injection), once a day for 5 d. Drug dosing solutions were prepared on the day of administration and were not reused. Seventy two hours after the final treatment, on day 35 post infection, all animals were euthanized, hepatic and splenic imprints were taken, fixed and stained and parasite burdens were determined by blinded counting. The total number of parasites per liver was calculated by multiplying the parasites per organ cell nucleus by 2×10^{5} by the weight of the liver in mg (Stauber, 1958). The unpaired student t test was used to compare the differences in response to Sb^{V} and miltefosine between arsenic exposure groups and control. Samples of the liver and spleens from all animals were prepared for analysis for both arsenic and antimony by ICP-MS.

2.14 Analysis of resistant lines *in vitro*

2.14.1 Extraction of genomic DNA from *L. donovani*

Twenty five ml of the relevant *L. donovani* cell line was cultured in a 75 cm^2 flask to 1 x 10^7 cells ml^{-1}. These cells were then centrifuged at 2600 g, 10 min, room temperature prior to incubation overnight at 56 °C in 450 µl TENS buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% (w/v) SDS) with 0.1 mg ml^{-1} proteinase K. Tris-buffered phenol/chloroform/isoamyl alcohol (PCI, 25:24:1) at pH 8.0 was used to extract DNA which was separated by centrifugation at 16,000 x g for 1 min at RT. The upper aqueous phase was carefully removed and used for further extraction with PCI and then CI (24:1) with the lower organic phase being discarded at each step. The extracted DNA was then precipitated using molecular grade 100% ethanol and washed twice in 70% ethanol to remove any inorganic contaminants. The DNA was then air dried and resuspended in 10 mM Tris- HCl (Qiagen), pH 8.5 and stored at 4 °C.

2.14.2 Scanning Electron Microscopy (SEM)

Glutaraldehyde was added directly to culture medium in a dropwise fashion to a final concentration of 2.5% (v/v) and fixed for at least 24 h at 4 °C. Subsequent preparation for EM was carried out by Mr. Martin Kierans, College of Life Sciences, Dundee. Cells were collected on Whatman nucleopore membranes and washed with 0.2M PIPES buffer (2 x 15 min) at pH 7.2. The membranes were then incubated for 16- 18 h in 0.2% aqueous osmium tetroxide and washed with distilled water (2 x 15 min) prior to dehydration through a graded ethanol series. Critical point drying was performed using a BAL-TEC CPD 030. Aluminium stubs with carbon adhesive tabs were used to mount membranes which were

then coated with Au/Pd using a Cressington 208 HR sputter coater. A Philips XL 30 ESEM or a Hitachi S- 4700 SEM operating at an accelerating voltage of 15kV was used by Martin Kierans and me to examine the samples.

2.14.3 Western blotting

Whole cell protein lysates were prepared from cell culture by centrifugation, re-suspended in Laemmli buffer (Biorad) containing 0.5% (v/v) 2-mercaptoethanol and boiled for 10 min prior to separation by SDS PAGE gel against a SeeBlue® Plus2 marker (LifeTechnologies). Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol: Towbin et al 1979) was used to equilibrate unstained SDS-PAGE gels. Gels were either stained using the Coomassie stain (40% (v/v) methanol, 10 % acetic acid and 0.1 % (w/v) Coomassie Brilliant Blue R250) and destaining solution (20% v/v methanol and 10 % acetic acid) or proteins were transferred onto Protan™ nitrocellulose membrane (Whatman) for 25 min using Trans-blot ® SD semi-dry electrophoretic transfer cell (Bio-Rad) at 25 V. Membranes were incubated for at least 1 h with 5% (w/v) dry milk in PBS containing 0.5% (v/v) Tween ® 20 to block non-specific binding sites. Following this blots were incubated for 2 sequential 1 h periods with primary and then secondary horseradish peroxidase (HRP)-conjugated antibodies in PBS containing 1% (w/v) dry milk and 0.5% (v/v) Tween ® 20, with an intermediate washing step (3 x 10 min) in antibody dilution buffer. Membranes were then washed (3 x 10 min) in buffer without milk and incubated with Amersham ECL™ detection reagent prior to exposure to Hyperfilm ECL™ (GE Healthcare).

2.14.4 Thiol analysis

From late log phase culture, triplicate samples of 5 x10^7 cells from each *Leishmania* cell line, were centrifuged at 3500 rpm for 10 min at 4 °C, washed in ice cold PBS and spun down briefly in an Eppendorf bench top microfuge at 4500g for 3 min. The supernatant was carefully aspirated and 50 µl of HEPPS buffer (40 mM HEPPS, 4 mM diethylenetriaminepentaacetic acid, pH 8) added followed by 50 µl of 2 mM monobromobimane and the pellet was resuspended. The sample was then heated for 3 min at 70 °C, cooled briefly on ice prior to the addition of 100 µl of 4 M methanesulphonic acid (pH 1.6) (Shim and Fairlamb, 1988). Following a further 30-min cooling period on ice the samples were centrifuged (14,000 rpm, 10 min) and the supernatant collected and stored at -20 °C prior to analysis. Acid-soluble thiols were separated by ion-paired, reverse phase HPLC on an ion paired Ultrasphere C^{18} column using a Dionex Ultimate 3000 instrument fitted with a Dionex RF-2000 fluorometer.

Section B: Field work

2.15 Ethics

Ethical clearance was obtained from Banaras Hindu University in Varanasi, the Kala Azar Medical Research Center (KAMRC) in Muzaffarpur, India and the University of Dundee, Scotland. Individual written consent was obtained from each patient (or their guardian) included in the study (Appendix A). Any person identified with symptoms of VL or post kala azar dermal leishmaniasis (PKDL) during the study was referred to an appropriate health care facility. Data from the arsenic analysis of tube well samples was referred to the

Public Health and Education Department, which is running the arsenic mitigation programme in Bihar.

2.16 Study area

The study took place in Mahudin Nagar block in the Samastipur district of the state of Bihar, India. Bihar is the third largest state in India and has the lowest literacy rate. Mahudin Nagar block lies just north of the River Ganges and has a mainly rural population of 184,521 (Census Organization of India, 2011). It is known to be endemic for VL; between 2006-2010, the average reported yearly incidence of VL was 7.78 per 10,000 population (Source: District Malaria Office records, Patna, Bihar) and Samastipur district, in which it lies, has been mapped as an area with 'high' levels of resistance to SSG (Sundar, 2001). A survey performed by the School of Environmental Studies, Jadavpur University in 2005 identified over 40% of the wells surveyed in this district to have arsenic levels above the World Health Organization (WHO) recommended limit of 10 μg L^{-1} (School of Environmental Studies, 2002).

2.17 Study design and population

A retrospective cohort design had to be employed because SSG treatment for VL is no longer recommended for routine use in Bihar (Sundar *et al*., 2008). We identified VL patients treated with SSG between 2006 and 2010 in the block of Mahudin Nagar from two sources. First, in January and February 2012, potential SSG treated subjects were identified from the VL patient register at the Primary Health Centre (PHC) in Mahudin Nagar and were searched for in their listed villages, at least twice, for inclusion in the study. Second, additional VL patients were identified in the visited villages. The patients were included in the study if they gave a clear history of having been treated with SSG (see below) and at

least one of the SSG treatments fell inside the study recruitment period (e.g. 2006 to 2010). Patients were excluded if they did not receive SSG treatment or if their SSG treatment was terminated due to unavailability of SSG.

The study subjects were visited in their own home. A modified form of a previously validated questionnaire was used (Hasker *et al*., 2010) to gather information on age, sex, caste, VL symptoms, health seeking behaviour, treatment(s) and response from the time of first onset of VL symptoms to the time of the field study (Appendix B). Additional data was collected on other illnesses, prior VL in the family and water sources used (e.g. local tube wells). The interviews were performed by experienced field workers. If the patient was a minor at the time of VL, his/her guardian was interviewed and if the patient had passed away or was unavailable due to relocation for marriage or work, then a relative was interviewed instead. The household tube well and local tube wells were geo-located by the research team using a GPS. The household tube well coordinates were used to identify patients' location (e.g. within or outside Mahudin Nagar town).

Clinical records, either the patient's own documents or those held at the PHC, as well as data gathered during the interviews were reviewed by an experienced physician to ascertain VL cases and treatment. In absence of treatment records, the information provided by patients or relatives allowed identifying the type of treatment received as the 3 main drugs for VL are given via different routes: (1) SSG is an intramuscular injection usually administered for 30 days in the lateral upper thigh or buttock area; (2) amphotericin B is given via an intravenous drip in the forearm or hand for either 5 days or 30 days dependent on formulation used and (3) miltefosine is administered for 28 days as a capsule for oral use.

Patients were classified into the following treatment outcome categories: success, no clinical improvement, relapse, death and toxicity taking 6-month cut off time from end of treatment as reference, in accordance with WHO guidance and VL clinical trial protocols (Sundar *et al*., 2000). 'Treatment success' was defined as patients who received SSG treatment and had not required another VL treatment within 6 months. 'No clinical improvement' were patients who experienced no change or a worsening of their original symptoms during or by the end of a full course of treatment who required a further VL treatment. This subgroup included patients who reported a treatment course of 60 days or more which is 2 times the recommended SSG treatment duration. 'Relapse' were patients who experienced a return of signs or symptoms of VL, for which they required further treatment, after initially having experienced a return to health following a course of treatment. If the outcome was 'Death', a verbal autopsy was carried out by the physician on the field team to ascertain if the subject died directly as a result of VL, (Appendix C). 'Toxicity' were patients whose treatment was terminated due to untolerated side effects. The term 'treatment failure' in this study covers all adverse outcomes that occurred within 6 months of VL treatment: no clinical improvement, relapse, death due to VL and toxicity.

2.18 Evaluation of arsenic exposure

2.18.1 Tube well water

The average arsenic concentration of the patient's main water source and 4 tube wells surrounding the patient's home was taken as an indication of the level of arsenic exposure for the patient. This exposure variable was chosen instead of the arsenic concentration in the patient's primary tube well (Sohel *et al*., 2009) in the context of this study as many

patients reported that their current primary tube well had been inserted in the years after their first SSG treatment for VL.

At the time of interview samples were collected from these 5 tube wells in a 15 ml Falcon tube after pumping off water for two minutes. Water samples were preserved with a drop of concentrated nitric acid until analysis. Flow injection hydride generation – atomic absorption spectrometry (FI-HG-AAS) was used to quantify the total arsenic content in water samples at the School of Environmental Studies (SOES), Jadavpur University, Calcutta as described previously (Chakraborti *et al.*, 2003). A standard sample from the Environmental Protection Agency was used as a reference. The lower detection limit was 3 $\mu g\ L^{-1}$ of arsenic. For quality control, 25% of the primary tube well water samples were selected to represent the low (n=6 (<10 $\mu g\ L^{-1}$)), medium (n =15 (10-50 $\mu g\ L^{-1}$)) and high (n=9 (>50 $\mu g\ L^{-1}$)) arsenic concentrations detected. These samples were re-analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) at Aberdeen University using a method described previously (Williams *et al.*, 2007b).

2.18.2 Urine samples

At the time of field questionnaire patients were requested to provide a sample of urine of at least 5 ml. Urine samples were preserved with a drop of hydrochloric acid and kept in cold conditions.

Prior to analysis, the urine samples were centrifuged at 3000 rev min^{-1} for 10 min and filtered through a 0.45-μm membrane filter (Chromatodisc 25A) to remove the suspended particulate. The urine was then digested for total arsenic analysis: 1 ml of the urine sample was placed in a small Kjehldahl flask and to this was added Suprapur® acid (0.5 ml HNO_3, 0.2 ml H_2SO_4 and 0.2 ml $HC1O_4$). The sample was heated using a small

funnel at the top of a sand-bath until it became clear. If necessary, a 0.2 ml aliquot of HNO_3 was subsequently added. Again the sample was heated until fumes of SO_3 evolved. The solution was cooled and made up to a volume of 5 ml. Total arsenic in the urine samples, as stable urinary metabolites of arsenic, was measured using FI-HG-AAS against a standard reference material (SRM 2670) obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

For quality control, 18 (26%) of the available urine samples were selected to represent normal (n=9; 5-40 μg L^{-1}) and elevated (n=9; >40 μg L^{-1}) urinary arsenic levels detected. These samples were re-analysed by ICP-MS at Aberdeen University as in Section 2.18.1 (Williams *et al*., 2007b).

2.19 Data management

The average arsenic concentration in the 5 tube wells was dichotomised using the WHO threshold for arsenic (As) in water: >= 10 μg L^{-1}. The three outcome variables are: (1) SSG treatment outcome (primary outcome) and (2) all-cause and (3) VL mortality (secondary outcomes). For the primary analysis, VL patients were classified based on their SSG treatment outcome as “treatment failure” or “treatment success” as described above. For the secondary mortality analyses, the study subjects were classified as “alive” or “dead” at the time of the field study. A sub-group identifying those that died due to VL was created based on the data from the verbal autopsies (see above).

The urine arsenic exposure variable was created with a binary cut off of arsenic contamination for a spot urine sample above the normal range of 5-40 μg L^{-1} (Mandal and Suzuki, 2002; Ravenscroft *et al*., 2009). In patients for whom it had not been possible to obtain a biological sample, an imputed value for their urinary arsenic level was calculated,

using the arsenic water level variable described above, in a regression prediction model controlling for age, sex and geographical location.

Data from the field interviews were double entered in a Microsoft Excel database independently by 2 data entry operators. The covariates included in the analyses are shown in Table 3.7. Briefly, age was split into 3 categories (e.g. < 5, 6-15 and > 16 years old) and SSG treated patients were dichotomized based on their "treatment course" using the duration of 30 days recommended by WHO (World Health Organization, 2010). Patients were classified based on their location (e.g. in or outside of Mahudin Nagar town), the "time to treatment" (e.g. < 12 weeks and ≥ 12 weeks in accordance with previous literature (Rijal *et al*., 2010)) and "the place of treatment" (e.g. government or private facilities). Finally, patients were classified into whether they had family members treated for VL with SSG prior to the patients' VL episode.

2.20 Statistical analyses

2.20.1 Raw data

2.20.1.1 Tube well water

Dot plots of the log of arsenic exposure from tube well water were drawn for the outcomes (1) SSG treatment outcome, (2) all-cause mortality and (3) VL mortality and the median arsenic exposures were compared using Mann Whitney U test. Receiver Operating Characteristic (ROC) curves were used to evaluate the WHO cut off for arsenic in water (10 μg L^{-1}) against the primary and secondary outcomes (e.g. SSG treatment, all-cause and VL mortality). The Kappa index was used to evaluate the agreement between the arsenic

levels reported by the two laboratories (SOES and Aberdeen University) that analysed the quality control samples.

2.20.1.2 Urine samples

Dot plots of the log of urinary arsenic level and Mann Whitney U tests were used to describe the relationship between urinary arsenic level, as a continuous variable, and SSG treatment outcome. Receiver Operating Characteristic (ROC) curves were used to evaluate the cut off for arsenic in urine (40 μg L^{-1}) against the outcome of SSG treatment. The Kappa index was used to evaluate the agreement between the arsenic levels reported by the two laboratories (SOES and Aberdeen University) that analysed the quality control samples.

2.20.2 Logistic regression

2.20.2.1 Tube well water

A logistic regression model was employed to assess if arsenic contamination in the local environment increased the risk of treatment failure in SSG treated patients. First, each covariate was analysed against the main outcome using logistic regression. Then, a multivariate logistic regression model was built, using a forwards stepwise method, with three a priori selected variables ("forced" variables: age, sex and location). Any additional variables which had a p value of <0.2 in the bivariate analyses were evaluated in the multivariate model and retained if they had a p value of < 0.05. The results were presented as odds ratios (OR) and their 95% Confidence Intervals (CI).

2.20.2.2 Urine samples

The same method from Section 2.20.2.1 was applied to both the biological samples and the biological samples with the imputed data set (Section 2.20.1.2).

2.20.3 Survival analysis

Survival analysis methods were used to compare survival in subjects exposed to elevated (>= 10 μg L^{-1}) versus normal arsenic water levels. The time origin for the survival analysis was the reported start date of symptoms. The date of 01/02/12, mid date of field visits, was used as the censor date. First, a Kaplan Meier (KM) survival curve and the log-rank test were used to evaluate the association between arsenic in water and all-cause mortality. Cox regression was used to estimate the risk of all-cause mortality in patients exposed to arsenic contaminated wells while controlling for possible confounding factors. The proportional hazards assumption was tested using visual methods and on the basis of scaled Schoenfield residuals (Hess, 1995). If the proportional hazard assumption was invalid the effect of arsenic exposure was allowed to vary over time by fitting 2 time varying covariates in the model. The KM plots were used to determine the temporal periods in which the effect of arsenic changed. The final regression model included arsenic exposure, three a priori selected variables ("forced" variables: age, sex and location) and any variables that were associated with mortality as assessed by the log rank test ($p < 0.2$) and remained significant ($p < 0.05$) in the final model. Hazard ratios (HR) and their 95% CI were used to express the risk in the final model. Analogous analyses (e.g. KM and Cox regression model) were used to study the risk of VL mortality associated with arsenic exposure.

Chapter 3-
Results

Table 3.1 Sensitivity of *Leishmania* cell lines and macrophages to metalloids As and Sb

Data are weighted mean ± standard error of 3 separate experiments from late log growth phase, using triplicate measurements, except where indicated. The bracketed figures are the mean ± standard error of the hill slope.

Cell line	As^{III}, µM	Sb^{III}, µM	Sb^{V}, µM
LdBob amastigote clones†	8.21 ± 1.1 (2.45 ± 0.2)	355 ± 29 (2.82 ± 0.3)	40,000 ± 490 (1.53 ± 0.3)
LV9 promastigote clones†	21.5 ± 1.6 (2.49 ± 0.16)	139 ± 8 (2.14 ±0.13)	>40,000
BALB-c peritoneal macrophages	4.95 ± 0.37 (3.08 ± 0.2)	22.9 ± 5.9* (1.7 ± 0.4)	11,600 ± 160* (1.3 ± 0.2)
THP-1 monocytes	12.2 ± 0.9 (1.57 ± 0.8)	124 ± 8 (3.15 ± 0.5)	>8,210
THP-1 macrophages	31.9 ± 1.7 (2.18 ± 0.1)	182 ± 12 (2.61 ±0.22)	>8,210
NMRI peritoneal macrophages	3.22 ± 0.17* (2.61 ± 0.22)	24.6 ± 0.9 (2.34 ± 0.0)	>8,210
C3H peritoneal macrophages	3.91 ± 0.13* (3.39 ± 0.3)		

*Weighted mean ± standard error of 2 separate experiment of triplicate measurements

† Clones derived from LdBob and LV9 displayed similar sensitivities to the metalloids and are each grouped together.

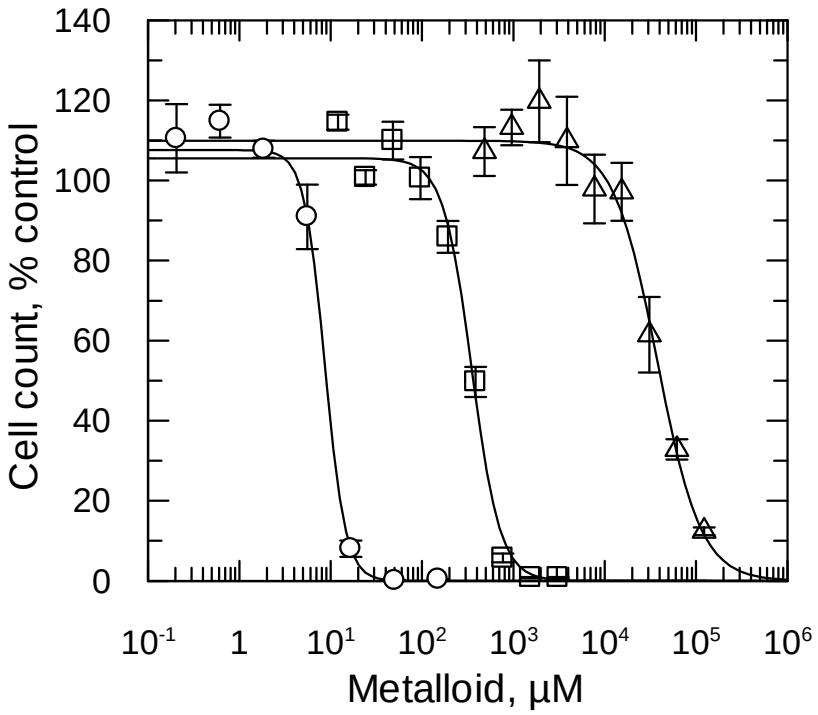


Figure 3.1 Susceptibility to metalloids in *Leishmania* axenic amastigotes

EC_{50} values for sodium arsenite (As^{III}, ○), potassium antimonyl tartrate (Sb^{III}, □) and sodium stibogluconate (Sb^{V}, △) respectively were determined against the LdBob clone LdSu3. The cells were axenically cultured in 96-well plates using a 72 hour resazurin-based assay. Curves are the nonlinear fits of data using the EC_{50} 3 parameter equation in GraFit. Data are the mean of triplicate measurements.

Section A: Laboratory work

3.1 Sensitivity of cell lines to metalloids

3.1.1 Sensitivity of *Leishmania* to metalloids

The axenic cell line LdBob was cloned and one of the clones was arbitrarily picked. To assess the relative potency of the metalloids against this clone LdSu3 the EC_{50} values of As^{III}, Sb^{III} and Sb^{V} were determined (Figure 3.1) and (Table 3.1). The order of EC_{50} values obtained for As^{III} (8.21 μM), Sb^{III} (355 μM), and Sb^{V} (40.4 mM) were in keeping with published data (Brochu *et al*., 2003; Goyard *et al*., 2003). However the LdBob clone is considerably more resistant to antimonial compounds than previously observed (Brochu *et al*., 2003; Goyard *et al*., 2003; Wyllie *et al*., 2004). The results obtained demonstrate the higher potency of the trivalent form of the elements and that arsenic, by molar comparison, is more toxic than antimony. Although the toxicity of Sb^{III} is similar between amastigotes and promastigotes (Table 3.1), Sb^{V} accumulates less in the promastigote form of *Leishmania*, than in the axenic amastigote form, and is thought to be pharmacologically inactive in the promastigote (Brochu *et al*., 2003; Wyllie *et al*., 2004). The finding that the EC_{50} of LV9 promastigotes is greater than 40 mM Sb^{V} supports this.

The high level of baseline sensitivity to Sb^{V} of the LdBobSu3 clone of 40.4 ± 4.9 mM warranted further investigation. At this concentration it was hypothesized that the mechanism of action for cell death may be ionic strength. Therefore the sensitivity of the Sb^{V} exposed clone was tested against sodium gluconate, a compound without an active anti-leishmanicidal ingredient. The EC_{50} of sodium gluconate was comparable at 24.3 ± 3.1 mM demonstrating that the Sb^{V} in sodium stibogluconate was not the

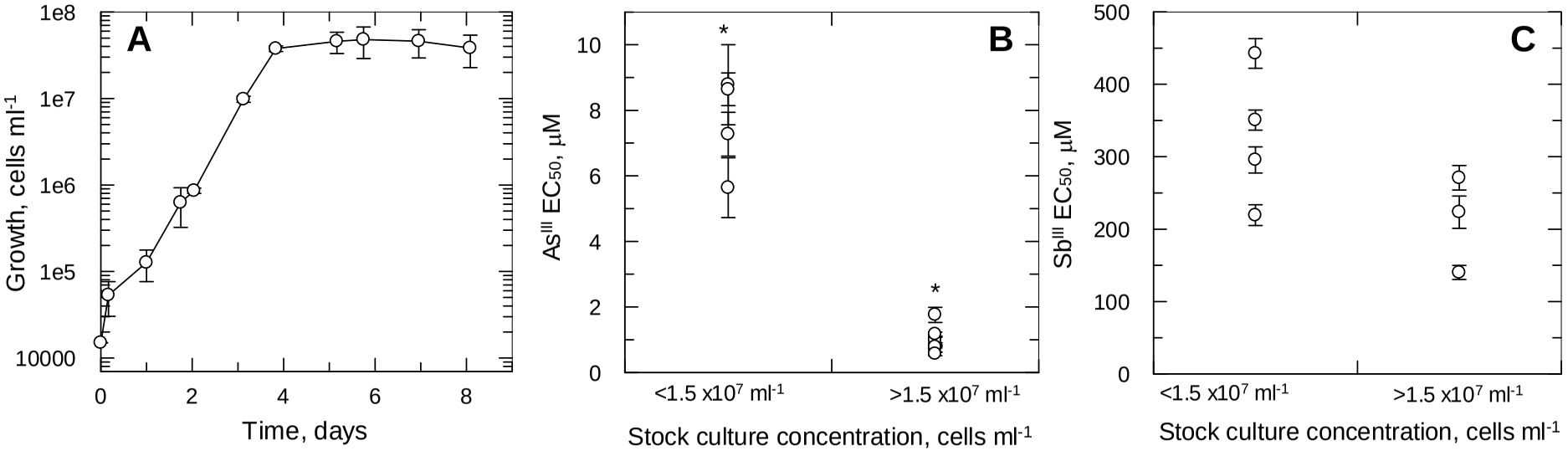


Figure 3.2 Growth curve and sensitivity of axenic amastigote clones to stock culture concentrations

Panel A: growth curve. Panel B: susceptibility to AsIII. Panel C: susceptibility to SbIII. Clones of LdBob were grown in axenic amastigote media (Table 2.1) and cell count enumerated manually with a haemocytometer, Panel A. Sensitivity to trivalent metalloids was determined by culturing axenic amastigotes of LdBob in media in 96-well plates containing varying concentrations of metalloid in a 72 h resazurin-based assay. The EC_{50}s obtained were compared in relation to the stock culture concentration the cells were drawn from, Panel B and C. *= significant difference between groups $p < 0.0001$.

leishmanicidal ingredient and this Sb^V appears to be pharmacologically inactive. The original LdBob stabilate was retested for sensitivity to Sb^V and had an EC_{50} of 1 ± 0.04 mM, comparable to the previously published value from this laboratory of 1.64 mM (200 μg ml^{-1}) (Wyllie *et al*., 2004). Unexpectedly, it transpired that the LdBob that had been continuously passaged for some time from which the clone LdSu3 was derived, was found to have lost sensitivity to Sb^V, with an EC_{50} in the same range of LdSu3. The reason for this loss of sensitivity is unknown but this finding is interesting in light of the controversies of the sensitivity of axenic amastigotes to Sb^V (Vermeersch *et al*., 2009; Wyllie *et al*., 2004).

The susceptibility of the axenic amastigote line to As^{III} was strongly affected by the density of the stock culture from which cells were taken (Figure 3.2); if the parasites had reached stationary phase, the EC_{50} for As^{III} was around 0.98 ± 0.37 μM whereas cells in late log were less sensitive with an average EC_{50} of 7.58 ± 1.5 μM ($p<0.0001$)(Figure 3.2B). This effect was present with Sb^{III} but there was not a significant difference between the 2 groups ($p=0.13$)(Figure 3.2C). All subsequent EC_{50}s presented in this study are on cells taken from late log phase stock cultures.

The relative toxicity of the metabolites of arsenic (Section 1.3.2) MMA^{III} and DMA^{III} were additionally assessed in the axenic amastigote line. Both metabolites were greater than 8 fold more toxic than As^{III} with EC_{50}s of 0.97 ± 0.08 μM and 0.34 ± 0.03 μM, respectively. This relative potency is in keeping with the literature on mammalian cells (Styblo *et al*., 2000) although MMA^{III} is generally thought to have a higher toxicity DMA^{III}. The difference seen in the axenic amastigote susceptibility may be due to the preparation of DMA^{III} which had to be purified in oil and may be contaminated with H_2S, a toxic compound in itself (personal communication Dr. A. Raab).

3.1.2 Sensitivity of uninfected macrophages to metalloids

The sensitivities of different lines of macrophages were determined to establish the difference between macrophage and parasite sensitivity and for development of the in-macrophage assay (Section 3.2). No difference in sensitivity to As^{III} between starch elicited and non-elicited NMRI mouse macrophages was detected (3.22 ± 0.17 and 3.10 ± 0.21 respectively). No significant difference between the species BALB/c, NMRI and C3H was detected (Table 3.1). However, when compared to BALB/c mouse macrophages, the laboratory line THP-1 were > 2 fold less sensitive to arsenic as monocytes and > 6 fold less sensitive to arsenic as differentiated macrophages (p=0.03) consistent with previous literature (Sakurai *et al*., 2004). This effect is present but non-significant with Sb^{III} (p=0.25). In view of the sensitivity of macrophages to As^{III} it was not possible to select for resistance in intracellular amastigotes or accurately study the effect of As^{III} on parasite growth in this *in vitro* setting.

3.2 Optimisation of in macrophage assay

The in-macrophage assay was developed to optimise the assessment of amastigote killing within the macrophage, particularly in reference to Sb^{V}. The initial comparison of parasite to macrophage ratios had the same result in all parasite and macrophage cell types – a ratio of 40 to 1 killed most of the macrophages present and a ratio of 2 to 1 gave only a low level and inconsistent infection. A ratio of 10 to 1 was chosen as optimal, in keeping with KP Chang's work on parasite macrophage interactions (Chang and Dwyer, 1978). Using peritoneal-derived mouse macrophages rather than differentiated THP-1 cells increased the number of macrophages infected with either form of *Leishmania* parasite. When using mouse macrophages there was no significant difference in infection levels whether the infection period was 4 hours or up to 16 hours.

Table 3.2 Parasite strain and media type affect *L. donovani* amastigote infection levels within mouse peritoneal macrophages

Starch elicited mouse peritoneal macrophages were incubated in different media at a ratio of 10:1 with different forms of the parasite for 16 hours, extracellular parasites were washed away and infected macrophages were incubated for 72 h prior to Giemsa staining and enumeration using a 100x oil immersion lens. Each figure represents the average no of amastigotes per macrophage having counted 100 macrophages per well.

Parasite	**Media**	**Amastigotes cell^{-1}**	**% infected**
LV9 promastigotes	10% FBS, RPMI	2.27	67
LV9 promastigotes	20% FBS, RPMI	3.3	71
LV9 promastigotes	10% FBS, M199 (+ adenosine)	0.46	32
LV9 promastigotes	20% FBS, M199 (+ adenosine)	0.16	11
LV9 *ex vivo* amastigotes	10% FBS, RPMI	13.1	95
LV9 *ex vivo* amastigotes	20% FBS, RPMI	12.7	94
LV9 *ex vivo* amastigotes	10% FBS, M199 (- adenosine)	2.33	65
LV9 *ex vivo* amastigotes	20% FBS, M199 (- adenosine)	4.23	89

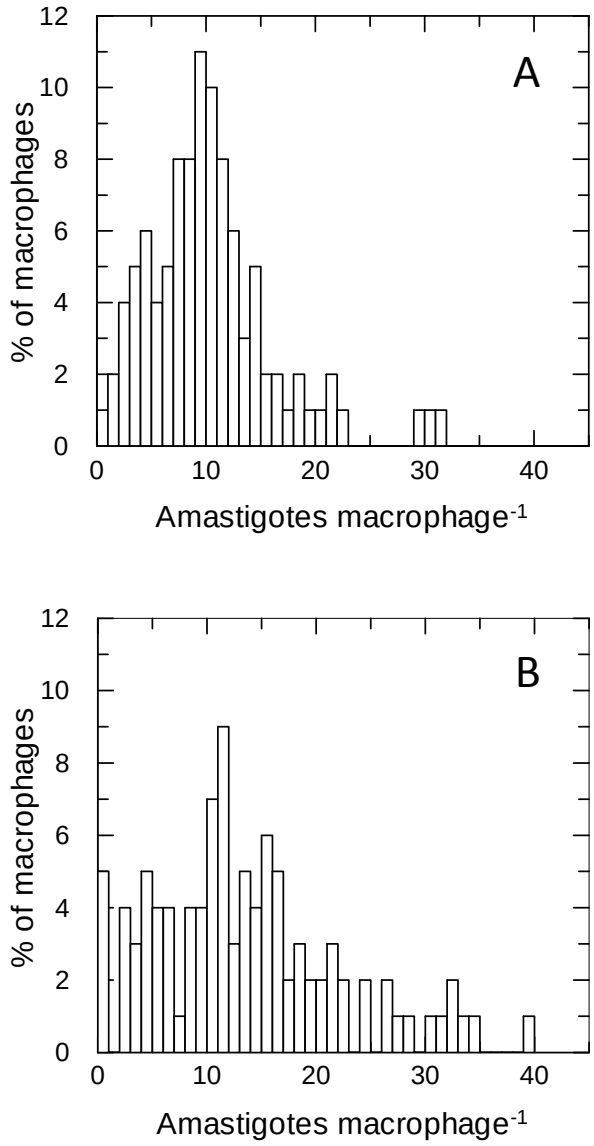


Figure 3.3 Histogram of amastigote growth post incubation period with macrophages

Starch elicited mouse peritoneal macrophages were incubated for 4 hours with *ex vivo* amastigotes after which all extracellular parasites were washed away and the macrophages were subsequently cultured in RPMI 20% FCS medium in chamber slides. Slides were fixed stained with Giemsa at 0 h (Panel A) and 72 h (Panel B) post incubation and the number of amastigotes per macrophage was enumerated manually using a light microscope with a 100x oil immersion lens.

At a ratio of 10 parasites to 1 mouse macrophage, *ex vivo* LV9 amastigotes were markedly more efficient than the laboratory line of LV9 promastigotes and axenic amastigotes at gaining entry to the macrophage (Table 3.2). Furthermore, it was important that the LV9 promastigotes were in late stationary phase (days 6-9) with visible dead or dying parasites, otherwise there was decreased infectivity and macrophage viability. This has previously been observed and explained by the presence of phosphatidylserine on the surface of these promastigotes helping to protect against host inflammatory responses (Wanderley *et al*., 2009). Another group has proposed that acidic preconditioning of promastigotes further improves infection levels (da Luz *et al*., 2009).

The composition of the medium also affected infectivity (Table 3.2). The type of medium used can have a profound effect on infection levels but there was no significant difference between using 10% versus 20% FBS supplementation. The high concentration of adenosine (100µM) in M199 media led to a considerable reduction in macrophage infection levels. The adenosine may have directly inhibited phagocytosis consistent with literature on macrophage function (Eppell *et al*., 1989).

It was found that intracellular growth and division of amastigotes had started to occur by 72 h in cell cultures with RPMI medium (Figure 3.3) but not in those with M199 medium. A growth study performed with time points 0, 24, 72 and 116 h demonstrated an initial lag phase in growth between 0 and 24h, minimal growth at 72h and doubling of amastigote levels by 116 h. This is consistent with studies on hamster macrophages and *L. donovani* (Chang and Dwyer, 1978).

The above results led to a final protocol using peritoneal mouse macrophages, a parasite macrophage ratio of 10:1 and an infection incubation time of 4 h with *ex vivo* amastigotes where possible (Section 2.7). The assay length chosen was 72 h as this did

Table 3.3 Susceptibility to metalloids in baseline and cloned resistant *Leishmania* axenic amastigotes

Leishmania axenic amastigotes were grown in flasks in the presence of varying concentrations of metalloid and a resazurin based assay was used for determination of EC_{50} values for As^{III}, Sb^{III} and Sb^{V} respectively. Clones were generated by limiting dilution of resistant lines growing well at the top concentration of the relevant metalloid. EC_{50} were calculated from 3 parameter equation in GraFit. Data are the mean of triplicate measurements. RF = relative factor in relation to baseline clone.

Cell line	As^{III}, μM	RF	Sb^{III}, μM	RF	Sb^{V}, μM	RF	Ratio Sb^{V}/Sb^{III}
LdBob original	nd		234.5 ± 27.1		1000 ± 40		4.3
LdSu3 clone	7.4 ± 0.1	1	239 ± 37	1	40,400 ± 4,900	1	169
Control line no drug	6.9 ± 0.6	0.9	246 ± 3	1	nd	-	
$LdSu3^{SbIIIR}$ clones	5.2 ± 0.3	0.8	230 ± 15	1	nd	-	
$LdSu3^{AsIIIR}$ clones	69.1 ± 5.2*	10	2383 ± 320*	10	nd	-	

* $p<0.05$ compared to baseline LdSu3 clone

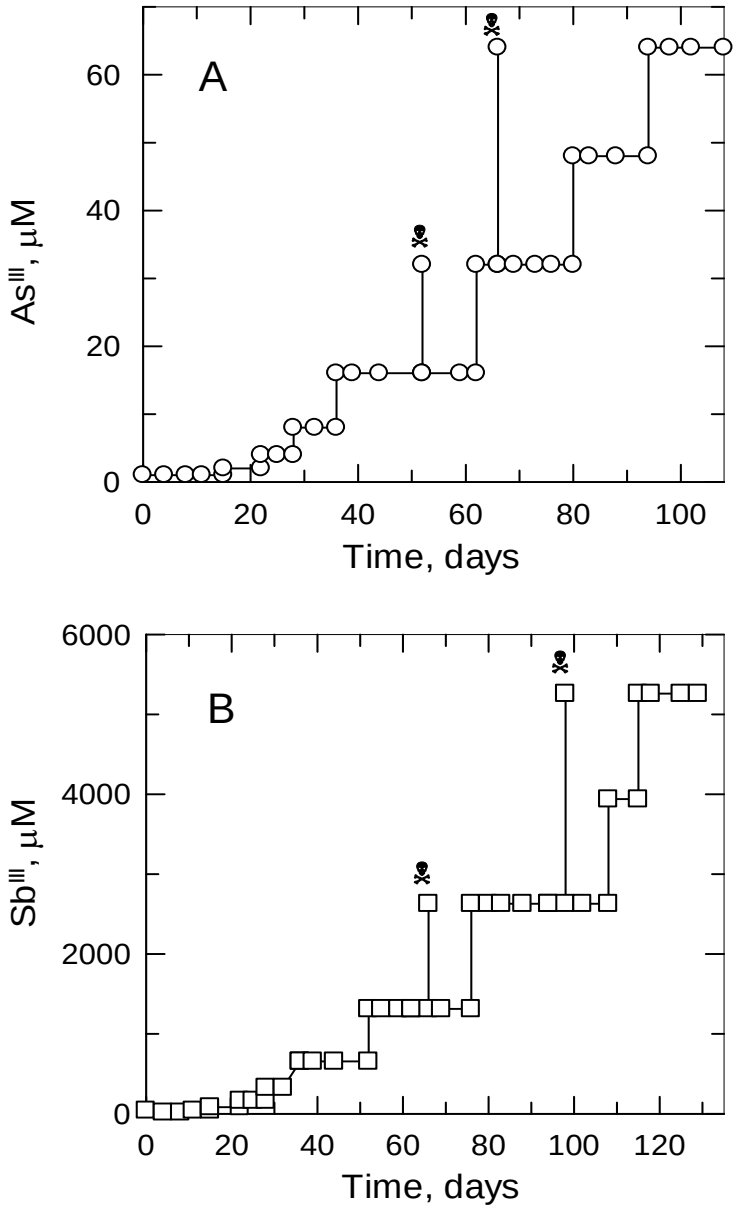


Figure 3.4 Generation of resistant lines of *L. donovani* to metalloids

Panels A and B represent exposure of LdBobSu3 to stepwise increasing concentrations of sodium arsenite (As^{III}, ○) potassium antimonyl tartrate (Sb^{III} , □), respectively. Each symbol represents a culture passage at that drug concentration. The growth rate at each culture passage was quantified using a haemocytometer.

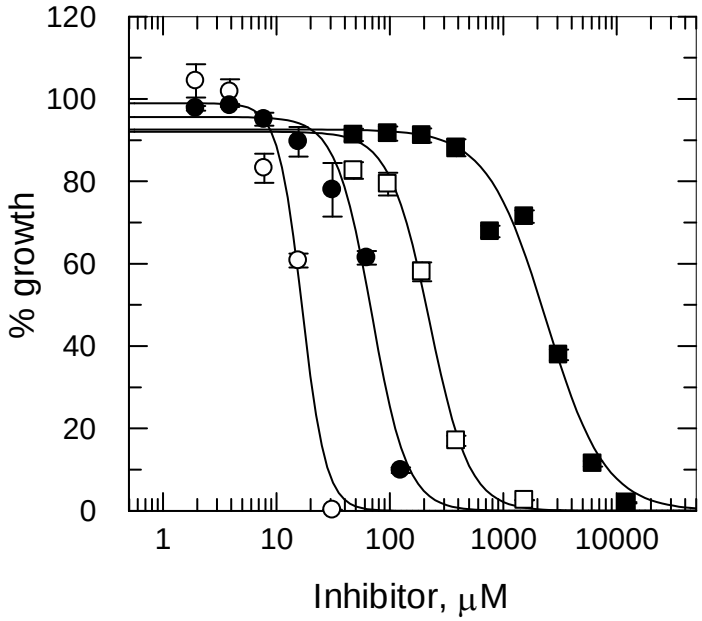


Figure 3.5 Susceptibility to trivalent metalloids in baseline and cloned As^{III} resistant *Leishmania* axenic amastigotes

Determination of EC_{50} values for sodium arsenite (baseline LdSu3, ○, As^{III} resistant LdSu3, ●) and potassium antimonyl tartrate(baseline LdSu3, □, As^{III} resistant LdSu3, ■) respectively was performed using a resazurin based assay with against axenically cultured LdSu3 and clone B7 of the generated As^{III} resistant LdBobSu3. The curves are the nonlinear fits of data using the EC_{50} 3 parameter equation in GraFit. Data are the mean of triplicate measurements.

not require a change of media. No significant difference was seen in Sb^{V} EC_{50} results between 72 and 120 h in incubations with no media replenishment.

3.3 Selection of drug resistant axenic amastigotes

A resistant line to the trivalent metalloids of Sb and As was generated in axenic amastigotes with the intention of performing a comparison between the associated mechanisms of resistance developed. This could potentially provide tools to test the hypothesis of parasite resistance selected for by exposure to arsenic present in the exposed population of Bihar. A clone of LdBob, hereafter LdSu3, was used. The starting concentrations were chosen to be approximately equal to the EC_{10} of the respective compounds, Sb^{III} and As^{III}. It took 125 versus 108 days respectively to produce a Sb^{III} ($LdSu3^{SbIIIR}$) and As^{III} ($LdSu3^{AsIIIR}$) resistant line with a normal growth rate at 64x the starting concentration (Figure 3.4). Large inoculums were required at high drug concentrations to maintain the culture.

The parasite line, $LdSu3^{SbIIIR}$, growing at 64x the starting concentration Sb^{III} was cloned by limiting dilution in the absence of drug. Assessment of sensitivity of the resulting $LdSu3^{SbIIIR}$ clones to As^{III}, Sb^{III} and Sb^{V} demonstrated that stable resistance had not been generated (Table 3.3), implying that tolerance to the metalloids was not retained through the cloning process.

Based on the above finding $LdSu3^{AsIIIR}$, was maintained at the top concentration of 64 μM As^{III} for 3 months prior to cloning. The As^{III} cloned lines showed approximately 10 fold resistance, with respect to baseline LdSu3, to As^{III} and Sb^{III} (Figure 3.5), (Table 3.3). There was no difference in sensitivity between control and clones to the control drug amphotericin B.

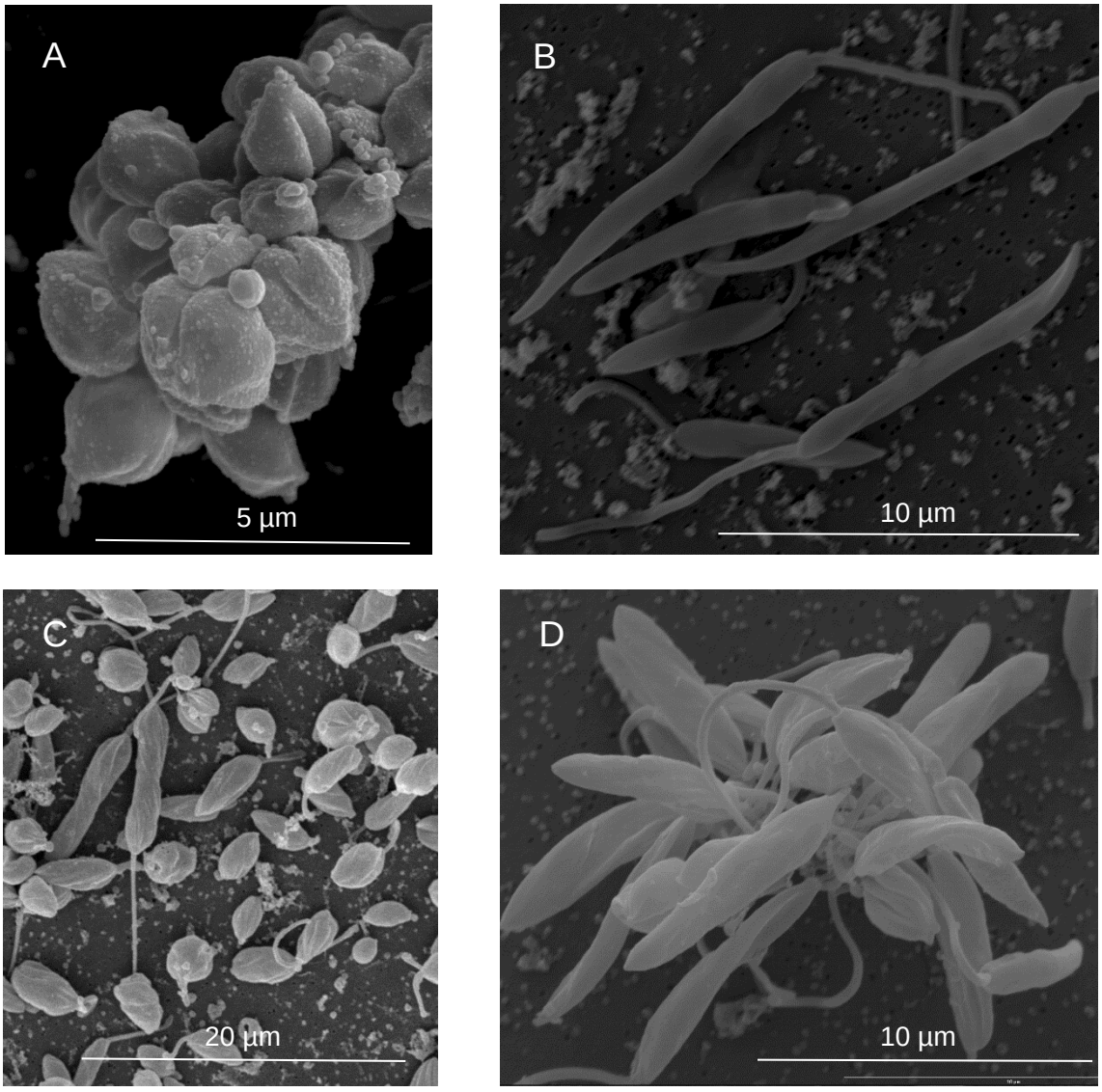


Figure 3.6 Scanning electron microscopy of LdBob and LdSu3

Log phase parasites were fixed using a final concentration of 2.5% glutaraldehyde and prepared for EM as in Section 2.14.2. Panels A and B represent the original LdBob clone in axenic amastigote and promastigote form respectively. Panel C represents the heterogenous population visible in the clone LdSu3 that was selected for As^{III} resistance. Panel D of LdBobSu3 cycled continuously in axenic amastigote form for > 120 d demonstrates classic rosetting usually only seen in promastigote cultures.

Table 3.4 Susceptibility to antimony species and intracellular thiol levels in *Leishmania* parasites exposed to Sb^V and control

A clone of LV9 was split into 6 lines, A-C that were exposed to increasing concentrations of Sb^V in macrophages and D-F that were passaged through non drug-exposed parasites. Following 3 passages, the sensitivity of the parasites to Sb^V was assessed using an in-macrophage assay (exposed vs unexposed groups: relative factor >9, $p<0.05$) and the sensitivity of promastigotes to Sb^{III} was assessed using a resazurin based assay (exposed vs unexposed groups: relative factor 0.86, $p>0.05$). Levels of intracellular thiols were measured in triplicate. On average, there was a 1.5 fold higher thiol levels in the exposed versus the non-exposed promastigotes ($p<0.05$)

		Intracellular amastigote	Promastigote		
Line from LV9 clone		**Sb^V, µM**	**Sb^{III} µM**	**GSH, nmol (10^8 cells)$^{-1}$**	**$T(SH)_2$, nmol (10^8 cells)$^{-1}$**
Sb^V exposed	**A**	> 4100	102 ± 6.7	2.62 ± 0.26	6.06 ± 0.33
	B	> 4100	88.4 ± 19	2.11 ± 0.17	4.98 ± 0.47
	C	> 4100	106 ± 2.8	2.50 ± 0.65	6.77 ± 0.14
No drug exposure	**D**	468 ± 200	107 ± 12.8	1.68 ± 0.45	4.69 ± 0.36
	E	436 ± 100	115 ± 10.6	1.27 ± 0.27	2.95 ± 0.23
	F	441 ± 40[†]	121 ± 5.8	1.61 ± 0.16	3.60 ± 0.33

[†] This EC50 was calculated using a 2 parameter equation as it was unable to be fitted to the 3 parameter model.

LdSu3 was cultured continuously in axenic amastigote conditions for up to 200 days during the selection of these resistant lines without cycling back to the promastigote stage. Under these conditions, the parasites in the control line, and the lines exposed to metalloids, developed a thinner, longer body, a visible flagellum and were observed to grow in culture in rosette formation. These are all features of the promastigote phenotype (Chang, 1979). In all cultures heterogenous populations are visible (Figure 3.6). Resistant lines have previously been created using axenic amastigote lines (El Fadili *et al*., 2009; El Fadili *et al*., 2005) but these morphological changes were not reported.

To assess whether this axenic amastigote line LdSu3 was sensitive to Sb^V within the macrophage it was tested in the in - macrophage assay at the same time as the $LdSu3^{AsIIIR}$ clone. Although both of the axenic lines were able to enter the macrophage, neither of them showed sensitivity to Sb^V within the macrophage.

In view of the above issues with the axenic line and experiments from other groups from our department showing that LdBob did not establish an infection *in vivo* no further experiments or analysis of this line were carried out.

3.4 Selection of Sb^V resistant *L.donovani in macrophage*

In order to select for resistance *in vitro*, in an environment as close to the conditions in humans as possible, resistance to Sb^V was selected for in amastigotes within macrophages using a modified version of a newly established method (Hendrickx *et al*., 2012). A clone of LV9 promastigotes recently isolated from BALB/c mice was passaged 3 times through Sb^V exposed (Lines A-C) and non-exposed macrophages (Lines D-F) (Figure 2.1). The resulting parasite sensitivities are summarised in Table 3.4. A stage-specific resistance was generated with no shift in promastigote sensitivity to Sb^{III} but intracellular amastigotes that had rapidly gained resistance to Sb^V up to 4

mM (relative factor >9). The speed of resistance selection and amastigote stage-specific resistance also occurred with the induction of paromomycin resistance using this method (Hendrickx *et al*., 2012).

Thiol levels measured in the parasite lines A-C and D-F were significantly different (Table 3.4) but only a 1.5 fold shift which would not fully account for the difference in sensitivity to Sb^{V} between the 2 groups. As the exposed and non-exposed lines have been selected for in triplicate they will be suitable for sequencing where known mechanisms of resistance will be assessed and changes in putative reductases can be assessed.

It is worth noting that the hill slope for Sb^{V} in macrophage assays is consistently shallow (range 0.4 to 0.6) in all experiments compared to axenic trivalent metalloid assays in amastigotes or promastigotes (range 1.5-4.5). This is likely to be representative of the need for activation of Sb^{V} and implies that this may be a rate limiting step. Additionally Sb^{V}, at the top concentration of 4.1 mM, never eradicated all the intracellular amastigotes. This may be due to the in macrophage assay used; longer exposures may result in more complete clearance of intracellular amastigotes.

It is important to establish where Sb^{V} is reduced – the macrophage, the amastigote or both and whether loss of this metabolic activation step is associated with Sb^{V} resistance. The current method of antimony speciation involves separation by HPLC coupled to ICP-MS (Hansen *et al*., 2011), a method not available in our laboratory. The indicators listed in Section 2.9.1 were tested for change in fluorescence in the presence of varying concentrations of Sb^{III} and Sb^{V} and the control compound Zn^{II}. There was no linear change in fluorescence of the indicators detected in the presence of 10 fold dilutions of Sb^{III} or Sb^{V} where a linear change for Zn^{II} was observed. The indicators were originally designed for divalent transition metal ions

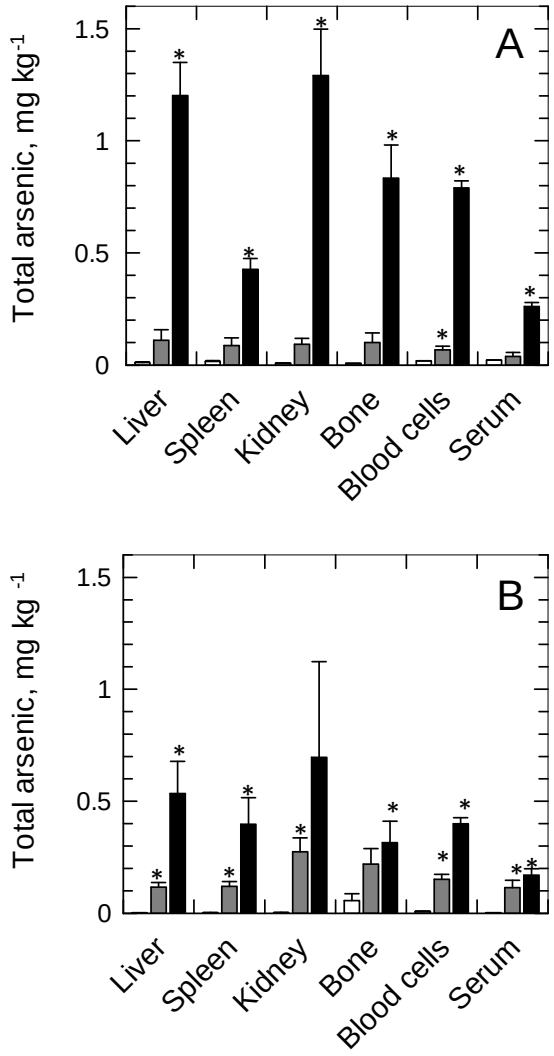


Fig. 3.7 Arsenic levels in organs of BALB/c mice.

At day 28 (A) and day 56 (B), three mice from each group (clear bars, 0 ppm; grey bars, 10 ppm; and black bars,

100 ppm) were culled, and liver, spleen, bone, and kidneys were harvested, homogenized, and digested before analysis of their total arsenic content by ICPMS (Section 2.X). In the case of spleen, kidney, and bone of the D28 control samples, these are the weighted mean of two independent analyses. Values are mean ± SE in mg per kg wet weight.

*$p < 0.05$ compared with 0-ppm group.

although have been demonstrated to work for the trivalent ions Tb^{III} (Terbium) and La^{III} (Lanthanum)(Figure 1.9). The hypothesis that this Sb^{III} may have a similar effect on fluorescence as these ions, has not, in my hands, been found to be the case.

3.5 Arsenic exposure in BALB/c mice

3.5.1 Arsenic exposure model

For *Leishmania* parasites to become resistant to antimony they require to be exposed to arsenic in the organs they reside in. To establish whether arsenic accumulates in the target organs, levels of total arsenic in liver, spleen, bone and blood, together with kidney as a marker of excretion, were measured at D28 and D56 of exposure to 0, 10 and 100 ppm of arsenic in their drinking water (Figure 3.7).

The mice in both 0 and 10 ppm groups gained weight at a similar rate and had a fluid intake of approximately 2 ml day^{-1}. In the 10 ppm group this is equivalent to 20 µg of arsenic $mouse^{-1}$ day^{-1}. The mice exposed to 100 ppm gained weight in a slower fashion and their fluid intake fell from 1 ml day^{-1} to 0.5 ml day^{-1} over the course of the 56 d, indicating cumulative toxicity. This fluid intake was equivalent to 50 – 100 µg of arsenic $mouse^{-1}$ day^{-1}.

Food intake was not directly measured but BALB/c ByJ mice are known to have a daily intake of 16g per 100g body weight (Bachmanov *et al*., 2002). The arsenic content of standard laboratory chow was measured to be 0.048 ± 0.005 µg g^{-1} which allows an estimation of the daily arsenic intake from food in each mouse of only 0.15 µg, a negligible amount.

Figure 3.7 shows that the concentration of arsenic in the drinking water of the mice correlated with the levels of arsenic detected in tissues. Of note, the levels of arsenic represented are from whole bone and may differ from those in the bone marrow

Table 3.5 Total arsenic and antimony in organs of BALB/c mice exposed to arsenic in drinking water

Arsenic levels were measured by ICP-MS in the livers and spleens of mice that have been exposed to 0, 10 and 100 ppm for 56 -63 days. The results represent the mean and standard error of measurements from 3 infected mice at passages 2, 3, 4 and 5 for liver and at passages 3 and 5 for spleen and from uninfected mice at 56 days. Total antimony results are mean and standard error of measurements from 3 mice from each arsenic exposure level at 5th passage, 72 h post sub-cutaneous injection of 50 mg kg^{-1} of Sb^{V}.

		Total Arsenic, mg kg^{-1}			Total Antimony, mg kg^{-1}		
		0 ppm	10 ppm	100 ppm	0 ppm	10 ppm	100 ppm
Liver	Infected	0.028 ± 0.011	0.12 ± 0.05	0.41 ± 0.09***	2.3 ± 0.1	2.2 ± 0.4	2.0 ± 0.1
	Uninfected	0.0020 ± 0.0006	0.12 ± 0.02**	0.53 ± 0.14**	ND [a]	ND	ND
Spleen	Infected	0.12 ± 0.08 (0.01 ±0.0004)[†]	0.23 ± 0.18	0.35 ± 0.28	2.4 ± 0.2	2.6 ± 0.1	1.1 ± 0.2*
	Uninfected	0.0026 ± 0.0007	0.12 ± 0.02**	0.40 ± 0.12*	ND	ND	ND

[a] ND= not determined

* p< 0.05, ** p < 0.01, *** p < 0.001 c ompared to control.

[†] Results of an independent experiment.

itself. However, the collagen of bone outer cortex is not cysteine rich so it is unlikely to hyperaccumulate arsenic.

In mice drinking 10 ppm of arsenic in their water the levels of arsenic in their tissues increased or were maintained at the same level between day 28 and day 56. In mice drinking 100 ppm of arsenic in their water their tissue levels fell reflecting the drop in their daily arsenic dose secondary to decreased fluid intake.

The main sites of arsenic metabolism and excretion are the liver and kidney and this is reflected in the elevated levels of arsenic in these organs (Vahter and Concha, 2001). Chronically exposed patients in Bangladesh were demonstrated on liver biopsy to have high levels of arsenic of between 0.5 and 6 mg (kg dry weight) $^{-1}$ (Mazumder, 2005). The conversion factor for wet to dry weight of mouse livers was calculated, from the freeze drying of three non-arsenic exposed BALB/c livers to be 3.53 ± 0.04. The levels of 0.4 to 1.5 mg (kg wet weight) $^{-1}$ seen over the 56 day time course in the 100 ppm group is thus equivalent to 1.41 ± 0.02 to 5.29 ± 0.06 mg (kg dry weight) $^{-1}$. These levels are similar to those found at similar arsenic exposure levels in C3H mice in the literature (Ahlborn *et al*., 2009; Kitchin and Conolly, 2010).

In Table 3.5 the mean arsenic contents of *Leishmania* infected and uninfected spleens and livers are compared at D56 of arsenic exposure. No significant differences were seen implying that that there is no interference in arsenic metabolism in mice due to the presence of *Leishmania*. Additionally, arsenic levels in the livers and spleens of arsenic exposed mice did not impact on the clearance of antimony by 72 h post Sb^V treatment, demonstrated by the similarity of antimonial levels in all mouse groups. The high standard error for the arsenic level in the non-exposed infected spleen in Table 3.5 is reflective of the small amounts of spleen available for analysis as most of the spleen was needed for isolation of parasites for drug sensitivity assays and further passage into

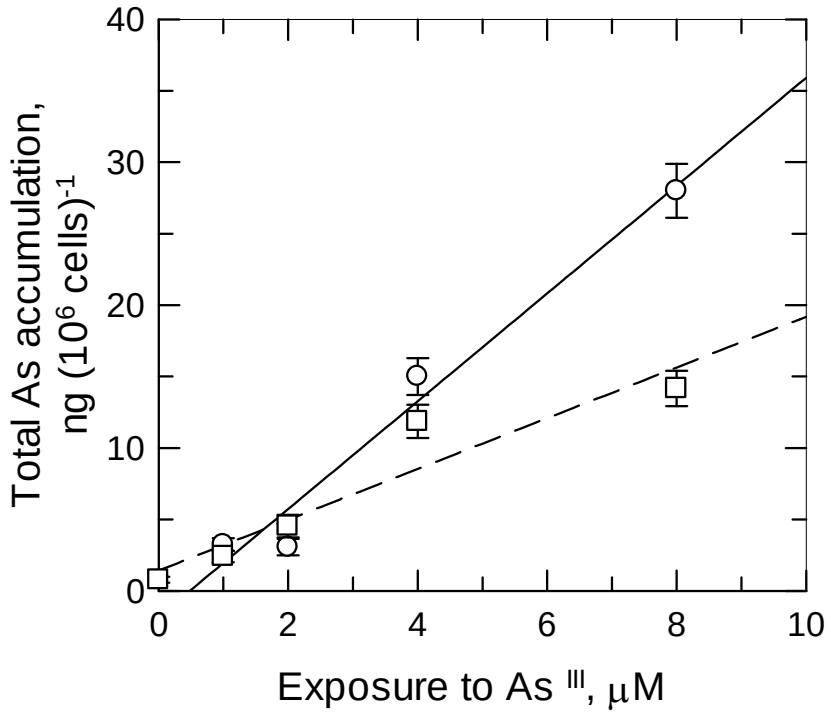


Figure 3.8 Accumulation of arsenic in mouse macrophages and THP-1 macrophages measured by ICP-MS

Differentiated THP-1 cells and *ex vivo* peritoneal macrophages were incubated for 72 hours with a range of As^{III} concentrations, removed, washed and their arsenic content measured by ICP- MS. The volume of 1 x 10^6 macrophages is equal 20μL as measured by Casey counter™. The degree of linear regression is related to the IC_{50} for mouse macrophages (O, 3.22 ± 0.17 μM) and differentiated THP-1 macrophages (□, 31.9 ± 1.65 μM)

mice. The low sample size and low arsenic content resulted in high variable count rates in ICP-MS analysis. ICP-MS analysis was repeated in an independent experiment using larger splenic samples from 3 infected mice with no arsenic exposure and the results are shown in Table 3.5 in parentheses.

3.5.2 Arsenic exposure and macrophages

Although it is known that antimony enters the macrophage (Berman *et al*., 1987) there is no experimental evidence of arsenic accumulation in macrophages in the literature which is important for this hypothesis. The current study shows a clear linear relationship between arsenic exposure *in vitro* and total arsenic content in-macrophage (mouse macrophage R-squared = 0.97, p= 0.002, THP-1 R-squared=0.90, p=0.014) that is macrophage dependent (Figure 3.8). In keeping with the difference in their EC_{50}s (Table 3.1) differentiated THP-1 cells accumulate arsenic less efficiently than mouse derived peritoneal macrophages.

The effect on peritoneal macrophages in mice exposed to arsenic at 0, 10 and 100 ppm was explored. There was no difference, in both mice with 28 and 56 day exposure, in the sensitivity of the macrophages from mice exposed to As^{III} in an *in vitro* resazurin assay when compared to macrophages from the control group (0 ppm 4.04 ± 0.32, 28 d, 10 ppm 3.82 ± 0.19, 100 ppm 3.00 ± 0.15, 56 d, 10 ppm 3.6 ± 0.19, 100 ppm 3.35 ± 0.11). However arsenic-exposed macrophages showed a trend towards a decreased ability to kill intracellular hamster *ex vivo* amastigotes using Sb^{V} (0ppm exposed macrophages 243 ± 59 µM, 10 ppm exposed macrophages 306 ± 49 µM, 100 ppm exposed macrophages 505 ± 93 µM).

Exposure to arsenic upregulates haem oxygenase which is cytoprotective (Wang *et al*., 2012). Its upregulation also leads to decreased levels of haem within the macrophage which decreases the activity of NO and NADPH oxidase (Alderton *et al*.,

2001). This could also leads to increased vulnerability of the intracellular amastigote, which is incapable of haembiosynthesis (Chang and Chang, 1985). The upregulation of haemoxygenase could explain why no difference was observed in sensitivity of the macrophages to arsenic. The trend in decreased efficacy of Sb^V against the amastigotes in arsenic exposed macrophages could be explained by the potential decreased activity of NO and NADPH as a result of decreased levels of haem. Other immunotoxic effects of arsenic on macrophage function may also play a role (Banerjee *et al*., 2009).

Measurement of the arsenic content of peritoneal derived macrophages in mice exposed to arsenic showed a significant difference in ng of arsenic $(10^6$ macrophages$)^{-1}$ between control and arsenic exposed groups (0ppm 0.01 ± 0.002, 10 ppm 2.39 ± 0.03, 100 ppm 2.24 ± 0.02). The injection of starch mobilises circulating blood monocytes and macrophages resident in the bone marrow to the peritoneum. This arsenic content, however, may not be reflective of the arsenic content of macrophages in the bone marrow as the cells will experience different arsenic exposures during migration and in residence in the peritoneum.

3.6 Selection of Sb^V resistance *in vivo*

3.6.1 Evaluation of a chronic visceral leishmaniasis model

Fifteen BALB/c mice were infected with an i.p. injection of 1×10^7 *ex vivo* amastigotes. On days 7, 14, 28, 56 and 110, 3 mice were sacrificed and parasite load quantified. Apart from an initial peak at D14 of 2×10^7 parasites per liver the parasite burden remained stable at 1.5×10^7 at all other time points. These parasites loads are similar to those reported in a methodological comparison study which favoured intraperitoneal injection over intravenous injection for homogeny of results (Rolao *et al*., 2004).

Table 3.6 Susceptibility *in vitro* to antimony species and intracellular thiol levels in *Leishmania* parasites previously exposed to arsenic *in vivo*

BALB/c mice were exposed to 0 ppm, 10 ppm and 100 ppm arsenic and infected with *L.donovani* (Figure 2.2). Harvested amastigotes from each group were differentiated into promastigotes. The sensitivity of the parasites to Sb^{V} was assessed using an in-macrophage assay (exposed vs unexposed groups: relative factor >7, $p<0.05$) and the sensitivity of promastigotes to Sb^{III} (exposed vs unexposed groups: relative factor of 1.5, $p>0.05$) and As^{III} (exposed vs unexposed groups: relative factor of 2.1, $p>0.05$) was assessed using a resazurin based assay. Levels of intracellular thiols were measured in triplicate. There was no significant difference in thiol levels between the 3 groups.

	Intracellular amastigote	**Promastigote**			
Arsenic exposure level	**Sb^{V}, µM**	**Sb^{III}, µM**	**As^{III}, µM**	**GSH, nmol (10^8 cells)$^{-1}$**	**$T(SH)_2$, nmol (10^8 cells)$^{-1}$**
0 ppm	590 ± 250	76.9 ± 2.2	15 ± 0.3	1.75 ± 0.20	5.03 ± 0.67
10 ppm	> 4100	85.9 ± 2.8	36.4 ± 0.5	1.52 ± 0.49	5.21 ± 0.90
100 ppm	> 4100	154 ± 3.4	27.7 ± 0.4	1.84 ± 0.10	4.65 ± 0.21

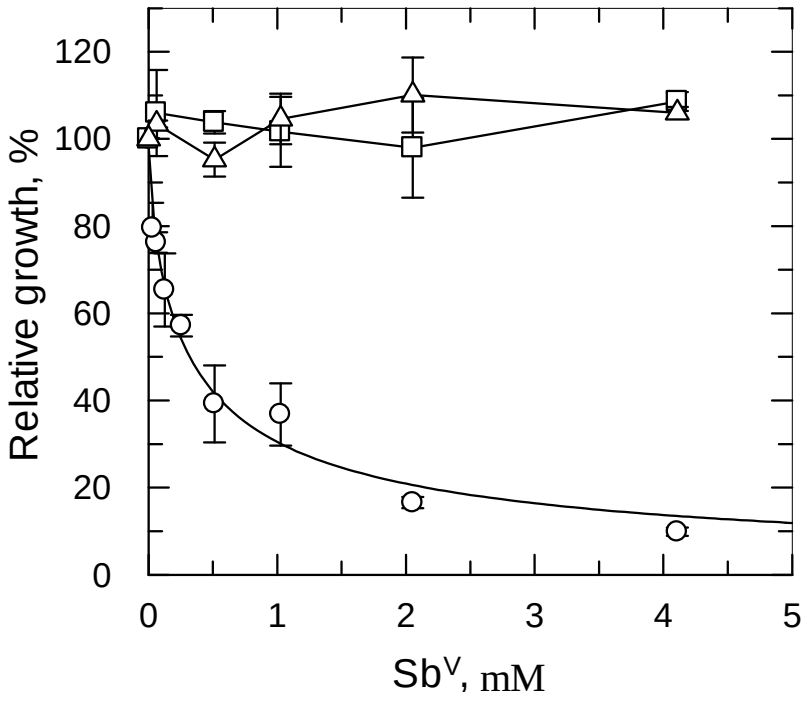


Fig. 3.9 Susceptibility of *ex vivo* amastigotes to Sb^V in-macrophage

BALB/c mice were exposed to 0 ppm (○), 10 ppm (□) and 100 ppm (△) arsenic and infected with *L.donovani* (Figure 2.2). Harvested amastigotes from each group were assessed for their sensitivity to Sb^V for 72 h within mouse peritoneal macrophages. Results from the 5th passage are presented here. Parasites recovered from the 10 and 100 ppm groups of mice remained insensitive to Sb^V at concentrations up to 4.1 mM. Amastigotes from the unexposed BALB/c mice had an EC_{50} value for Sb^V of 0.338 ± 0.058 mM.

3.6.2 Sensitivity of ex vivo amastigotes in *in macrophage* assay

The LV9 parasites exposed to arsenic within BALB/c mice for serial passages (Figure 2.2) gradually developed resistance to antimonial preparations. Following 3 passages, the parasites exposed at 100 ppm were already refractory to Sb^V at levels up to 4.1 mM (500 μg ml^{-1}). Those at 10 ppm were fivefold less sensitive to Sb^V compared to the control group at 0 ppm (0 ppm, 0.353 ± 0.049 mM (43 ± 6 μg ml^{-1}), 10 ppm, 1.87 ± 0.62 mM (228 ± 75 μg ml^{-1})). After the fifth passage, both groups of parasites exposed at 10 ppm and 100 ppm showed no response to Sb^V at 4.1 mM (relative factor >7) where the control group 0 ppm remained sensitive with an EC_{50} 0.317 ± 0.032 mM (41.1 ± 7.0 μg ml^{-1})(Figure 3.9). The resistance observed in the 10 and 100 ppm groups was retained following passage in mice not exposed to arsenic for 4 months. The EC_{50} in the control group of 0 ppm was 0.500 ± 0.19 mM (60.9 ± 24 μg ml^{-1}) at this time point.

Differentiation of *ex vivo* amastigotes proved problematic and several attempts were made using different media including NNN with rabbit blood. It was discovered that *ex vivo* amastigotes from all groups required supplemented RPMI medium to differentiate into promastigotes whereas *ex vivo* amastigotes from hamsters will differentiate easily in Grace's medium (Table 2.1). It should be noted that when the time this issues was resolved the parasite had been passaged in mice for 8 months in the absence of As^{III} pressure. The 0 ppm, 10 and 100 ppm promastigotes were tested for sensitivity to Sb^{III} and As^{III} and only small and non-significant shifts in EC_{50}s were observed (Table 3.6). Thiol levels in the promastigotes were measured but no difference was observed in levels of GSH or $T(SH)_2$ between the 3 groups.

When these promastigotes were used to infect macrophages in the in macrophage sensitivity assay the intracellular amastigotes from the 10 and 100 ppm groups were again refractory to Sb^V at 4100 μM (500 μg ml^{-1}) implying a stage-specific

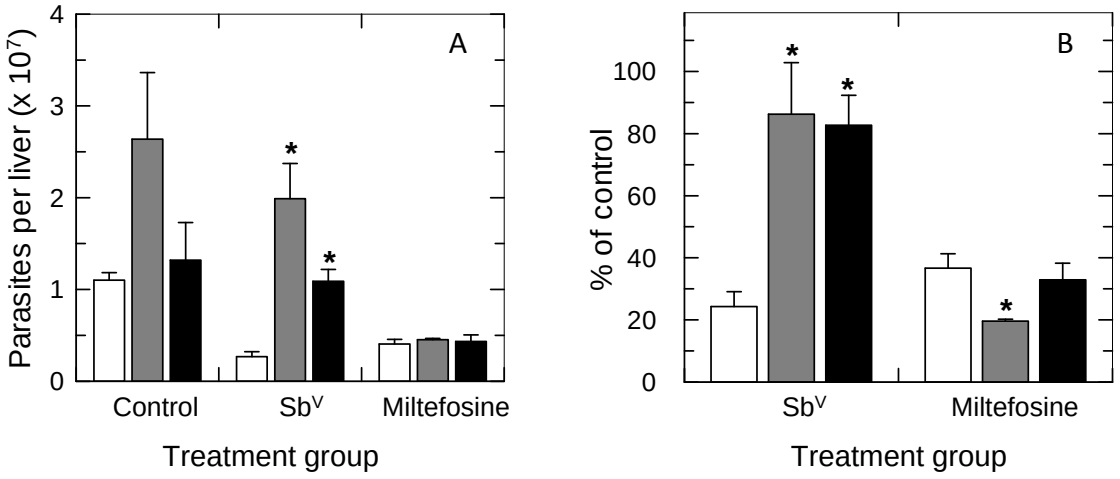


Fig. 3.10 Effect of drug treatment on the parasite burden of arsenic-exposed BALB/c mice.

Parasite burden is expressed as total parasite numbers per liver (A) and response to treatment as a percentage of the respective untreated control group (B). Following 28 d of infection, at each arsenic exposure level group (clear bars, 0 ppm; grey bars, 10 ppm; and black bars, 100 ppm), BALB/c mice were treated daily for 5 d with either s.c. injections of drug vehicle alone (n = 4, 5, and 5 at each arsenic exposure level), 50 mg kg^{-1} Sb^V s.c. (n = 3, 5, and 5), or miltefosine 12 mg kg^{-1} (n = 3, 4, and 4). Values are mean ± SE. *$p < 0.05$ compared with 0 ppm group.

resistance mechanism (Table 3.6). The 0 ppm group remained sensitive to Sb^V with an EC_{50} of 590 ±250 µM. Promastigotes from all groups were also resistant to the methylated pentavalent arsenic metabolite MMA^V at concentrations up to 15.4 mM (2500 µg ml^{-1}). In the in-macrophage assay the intracellular amastigotes from 0 ppm had an EC_{50} of 364 µM (59.0 ± 9.3 µg ml^{-1}) but the parasites from the 10 and 100 ppm group were refractory up to 1543 µM (250 µg ml^{-1}). MMA^{III} is the most potent of arsenic metabolites and these results suggest that the MMA^V reductase may have been lost or downregulated as a protective mechanism.

The putative Sb^V reductase TDR1 is known to reduce MMA^V (Denton *et al*., 2004). The antibody against the putative reductase TDR1 generated by Graham Coombs lab was tested directly against *ex vivo* amastigotes that had previously been exposed to 0, 10 and 100 ppm as there is reported differential expression between phenotypic stages. No response to the TDR1 antibody was generated on Western Blot in any group despite a positive response to the control pteridine reductase-1 antibody.

3.6.3 *In vivo* assessment of Sb^V resistance

At the end of the fifth passage all groups of mice were treated with drug vehicle, Sb^V or miltefosine with ongoing arsenic exposure in drinking water. In the 10 and 100 ppm administration of Sb^V s/c at 50 mg kg^{-1} only reduced the parasite burden to approximately 80% of control whereas in the 0 ppm Sb^V achieved suppression down to 33.6% of control (Figure 3.10). Miltefosine had a similar effect on parasite burden in all groups (Figure 3.10, Panel A). In the 10 ppm group in Figure 3.10, panel B, there is a significant difference in % suppression of parasite burden compared to control but this may be related to the variability of parasite count within the control group. This difference could also be attributed to arsenic's immunosuppressive effect, as part of the mechanism of action of miltefosine is to increase the sensitivity of the macrophage to

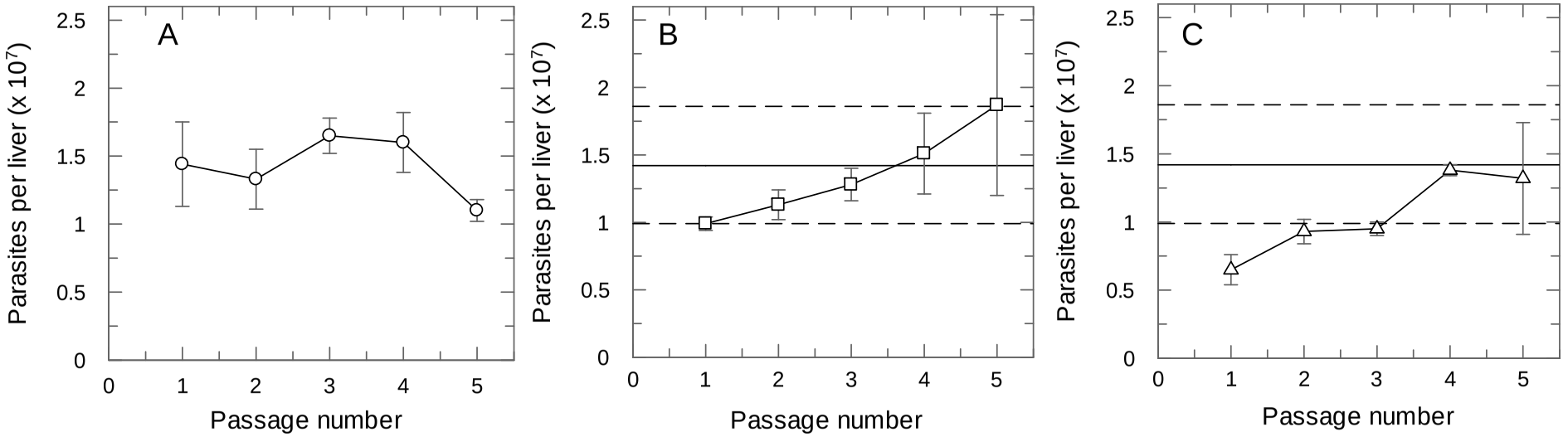


Fig. 3.11 Effect on parasite load of successive passages in BALB/c mice exposed to arsenic

BALB/c mice were exposed to 0 ppm (Panel A, ○), 10 ppm (Panel B, □) and 100 ppm (Panel C, △) arsenic and were infected with an i-p injection of 1 x 10^7 *L. donovani.* Following a 28 day infection parasite load was quantified by counting the number of parasites per 500 hepatic cell nuclei on Giemsa stained hepatic smears. Parasite burden is expressed as total parasite numbers per liver. The horizontal solid and dotted lines in Panels B and C represent the mean ± confidence interval of the parasite load of the non-arsenic exposed group, 0 ppm, across the 5 passages.

IFN-γ receptors (Dorlo *et al*., 2012). This latter explanation is less likely though as a significant difference in miltefosine sensitivity was not observed in 100 ppm group. The ineffectiveness of Sb^V at reducing the parasite load demonstrated that the cross-resistance between arsenic and antimony is active *in vivo* as well as *in vitro*.

3.6.4 Effect of arsenic exposure on parasite load

When parasites are passaged in the presence of 10 or 100 ppm there is a clear trend of increased parasite burden in the liver over multiple passages (Figure 3.11). The large error bars at the end of the 5^{th} passage represent the presence of amastigote clumping on the hepatic smears in the arsenic exposed groups making the parasite load difficult to quantify.

Although the trend is visible in the 10 ppm group the variation falls within the mean ± confidence interval of the non-arsenic exposed group. In the 100 ppm group, as noted in Section 3.6.1, the mice did not gain weight as rapidly as the control group. This increase in parasite load to levels similar to the 0 ppm group is in the context of consistently smaller liver (0 ppm 950 ± 52 mg, 100 ppm 688 ± 68 mg). This change in parasite burden may represent an initial treatment effect of the arsenic exposure which is overridden as the parasites develop resistance (Section 3.6.2 and 3.6.3).

Aliquots of 10^8 *ex vivo* splenic amastigotes from each group were taken at the end of the second passage and measured for their total arsenic content. The results confirmed that the arsenic content was higher in the mice exposed to arsenic at 10 and 100 ppm. Calculations of the intracellular arsenic content of 0, 10 and 100 ppm exposed amastigotes gave values of 8 ± 0.5, 44.0 ± 2.2 and 57.3 ± 1.6 µM. It would have been useful to track this but the amastigotes at the end of each passage were needed for re-infection and for assessment of sensitivity to Sb^V in the in-macrophage assay.

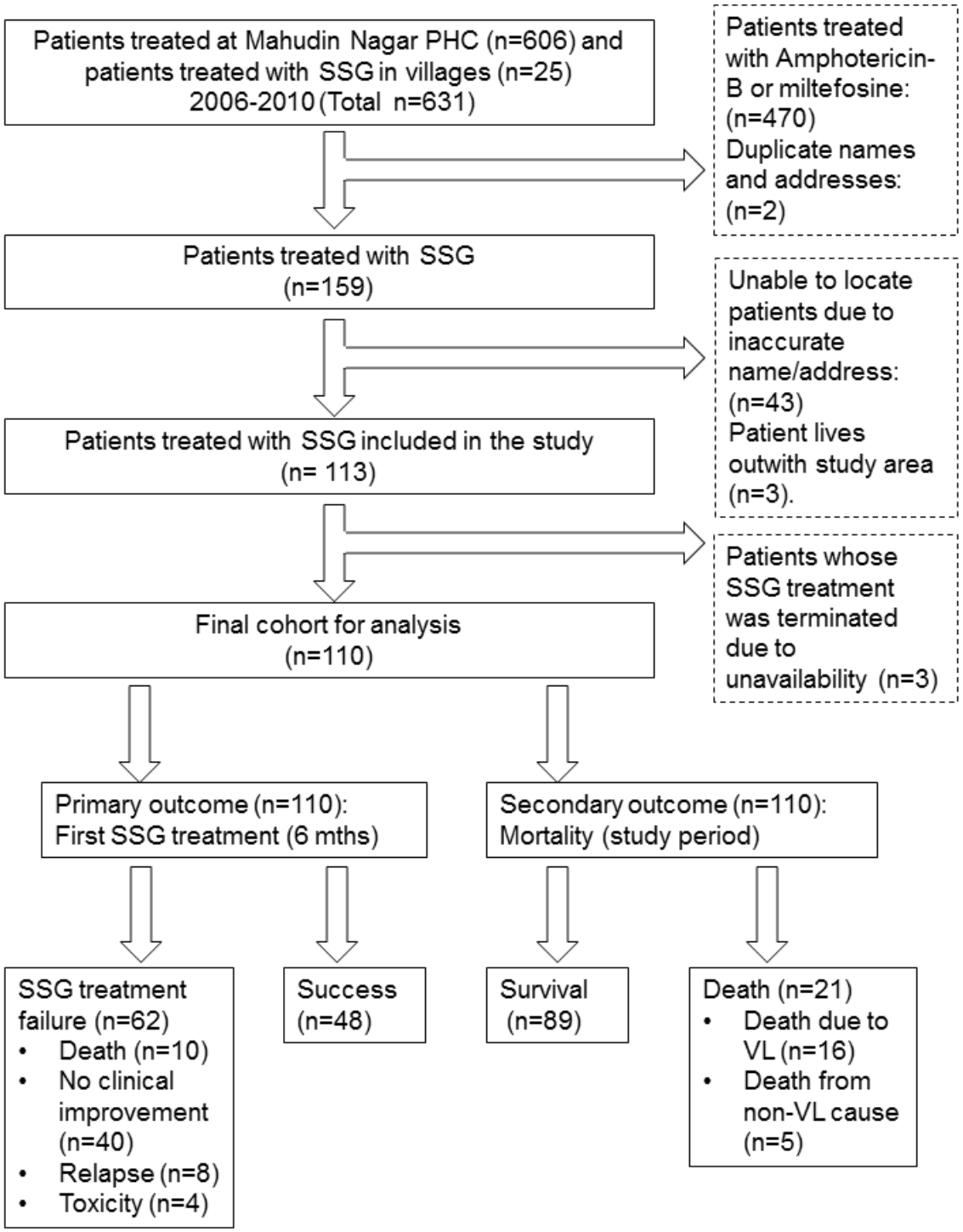


Fig 3.12 Flow chart of individuals identified as having received SSG treatment between 2006 and 2010

Additional outcomes: One patient had a still birth during antimonial treatment. One patient was identified with PKDL at the time of study.

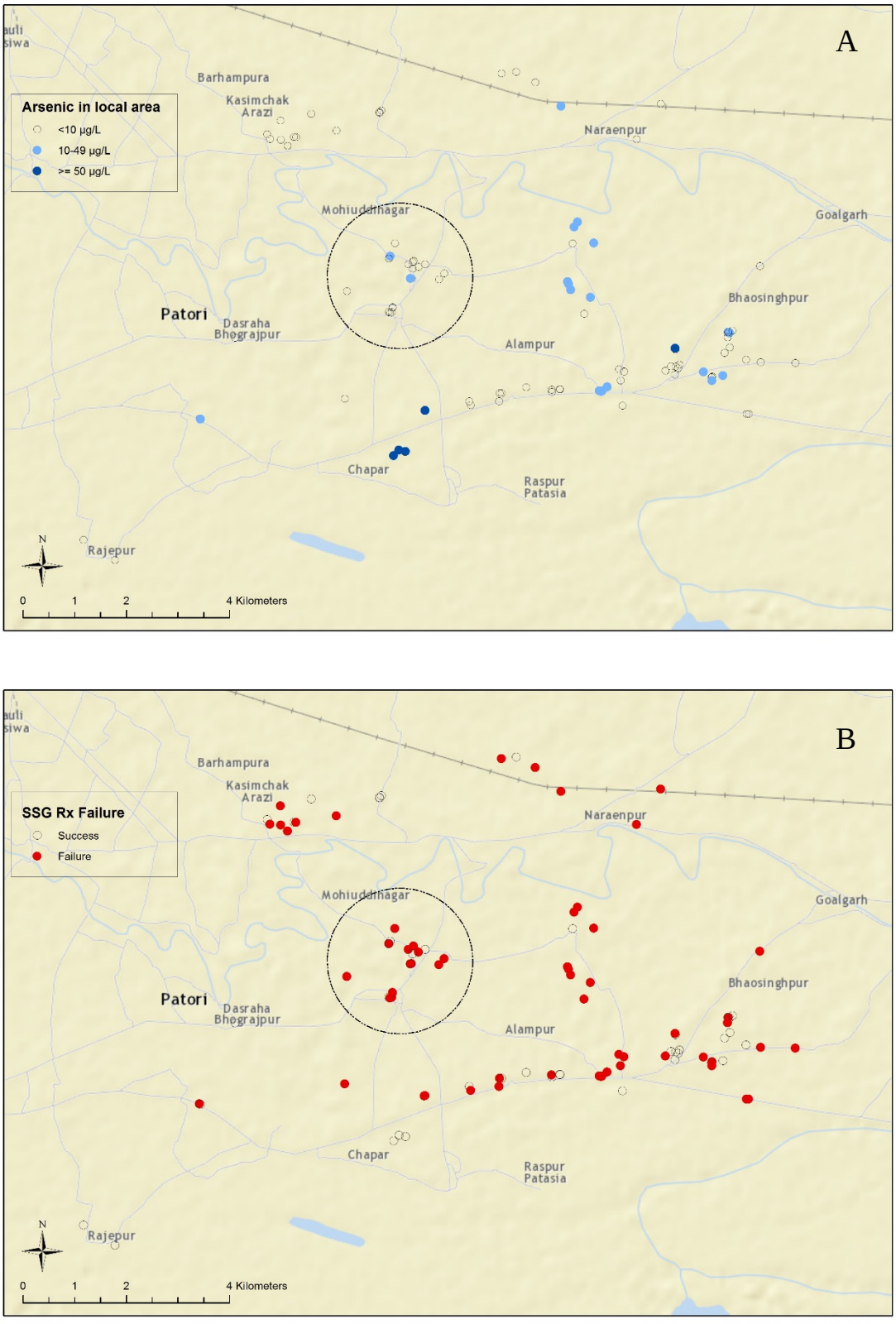
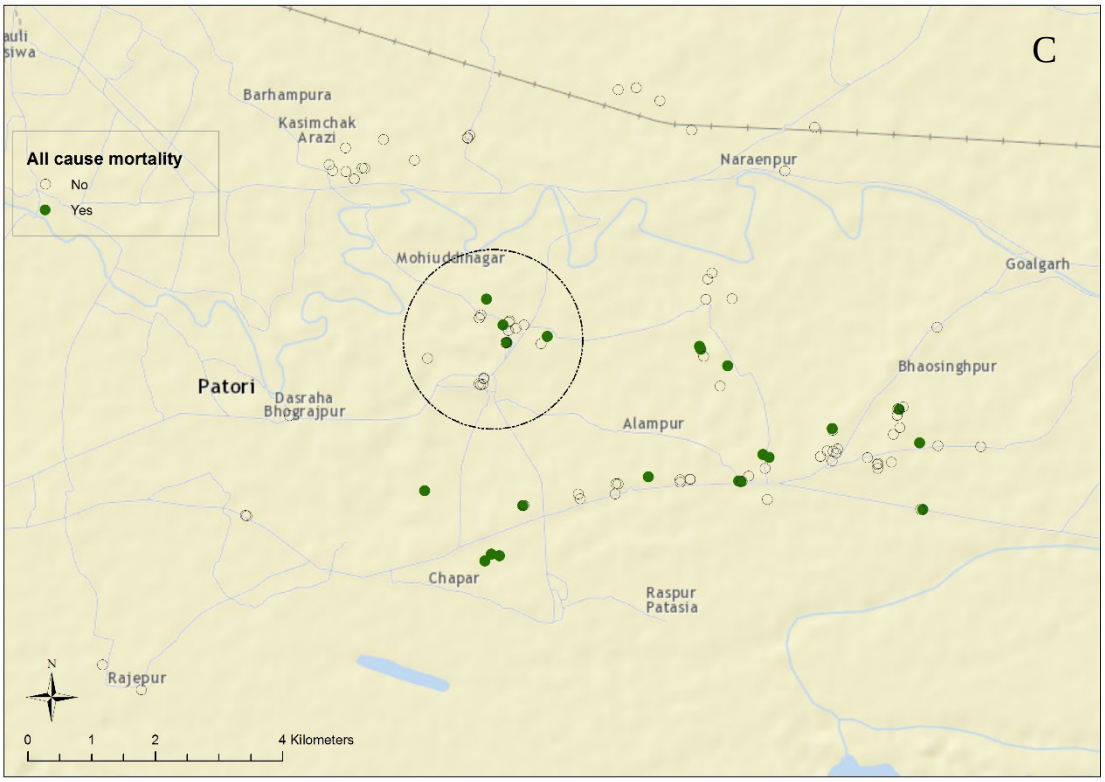


Figure 3.13 Map of Mahudin Nagar showing town boundary, distribution of arsenic contaminated wells (A), treatment failure (B), all-cause (C) and VL mortality (D)

Arsenic levels represented are the mean of water samples taken from 5 wells in the patient's local area.



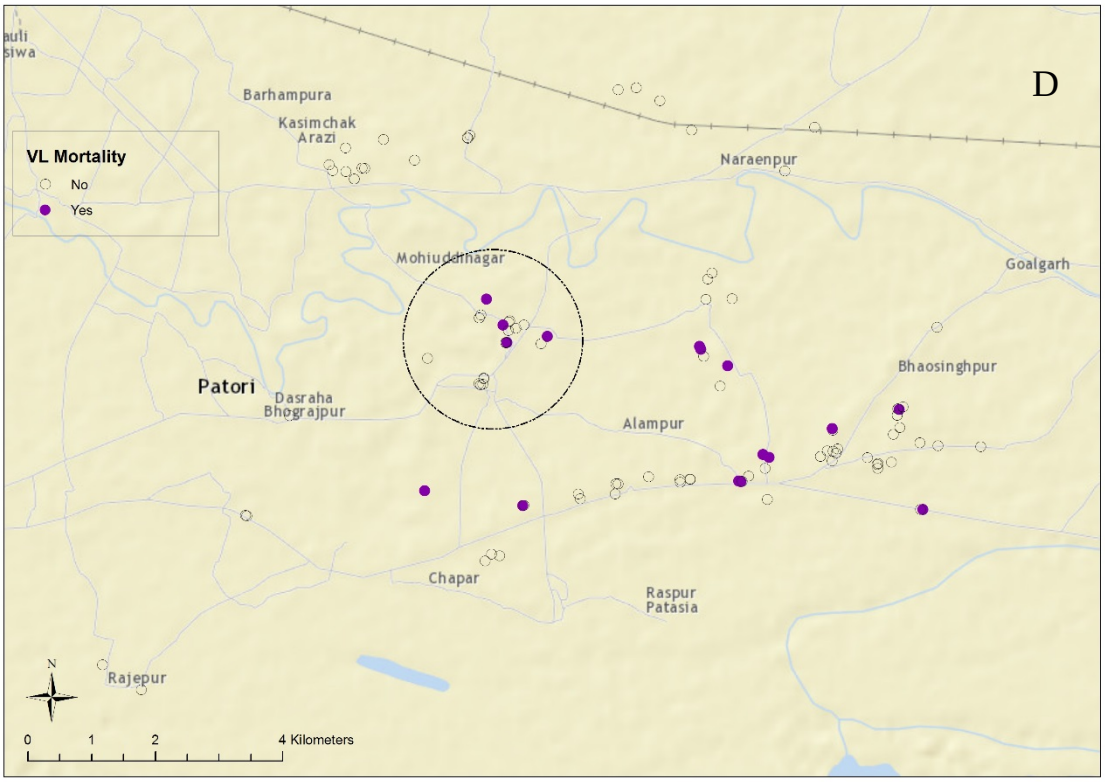


Figure 3.13 continued.

Section B: Field Work

3.7 Study population

Six hundred and six patients were identified that were treated for VL from January 1st 2006 to December 31st 2010 at the PHC of Mahudin Nagar (Figure 3.12). One hundred and thirty four (22%) of them were treated with SSG. The rest (n=472) were excluded from the study as they received miltefosine or amphotericin B (n=470) or were duplicate entries (n=2). Twenty-five additional VL patients treated with SSG from 2006 to 2010 were identified at the time of the fieldwork visits. Out of the 159 patients identified who had been treated with SSG, the houses of 113 (71%) of them were located. The reasons for being unable to locate patients included incomplete/inaccurate information (n=43) and distance from Mahudin Nagar block (n=3). From the 113 patients, 69 (61%) of them were present for interview. Twenty one (19%) of the subjects were dead and 23 (20%) were living outside the study area at the time of the visit. Relatives from the 44 missing subjects were interviewed. Based on the information gathered, 3 patients were excluded as they had their SSG treatment terminated due to unavailability of the drug. A cohort of 110 VL patients treated with SSG was finally included in the analyses.

3.8 VL cases and treatment

The 110 patients were aged between 3 and 60 years old, with a median age of 14 and a ratio of males to females of 3:2. Twenty percent of subjects lived within the area of Mahudin Nagar town (Figure 3.13). Twenty-three (21%) of patients had experienced other illnesses prior to their VL episode including the infectious diseases of malaria (n=2), tuberculosis (n=2), hepatitis/jaundice (n=4), cholera (n=1) and polio (n=1). Non-infectious illnesses included asthma (n=7), diabetes (n=1), arthritis (n=1), eczema (n=1)

and neurological complaints (n=3). All study subjects reported similar symptoms from their VL episode of fever, anorexia and weight loss (100%, 91%, 90% respectively) with malaise, abdominal distension and skin pigmentation being less common (72%, 44% and 7% respectively). Confirmation of the diagnosis of VL was available in the form of record of a positive rapid diagnostic test for VL (e.g. rK39 dipstick) in 26 subjects (24%). Parasitological confirmation was not available for any patient.

Seventy-one patients (65%) were only treated with SSG; 52 received one treatment, 18 received two and 1 patient received three SSG courses. Fourteen out of the 52 patients who received one treatment reported SSG treatment courses of 60 and 90 days duration which are 2 and 3 times the recommended duration. Thirty-nine patients (35%) were treated with miltefosine or amphotericin B before (n=3) or after (n=34) SSG treatment or both (n=2). The mean number of treatments (using any drug) received per person was 1.6 (±0.6).

The number of clinical documents confirming the treatments administered was limited. For example, only 16 treatment records were available for review out of the 85 patients treated at the PHC. According to those records, the treatment of 7 out of 16 patients (44%) did not conform to WHO recommendations – either by dose (only 10mg/kg) or duration (20 vs 30d). However, the treatment duration reported by the patient in the questionnaire matched the PHC treatment card only in 10 out of these 16 patients (63%). Information on doses was not available from the patients' interviews.

3.9 Outcomes of antimonial treatment

Sixty two (56%) of the study subjects were classified as "treatment failure" (Figure 3.13B): 10 (9%) died due to VL within 6 months, 40 (36%) patients experienced no

Table 3.7 Bivariate logistic regression analysis of risk factors for SSG treatment failure

Variable	Treatment failure n=62* (%)	Treatment success n= 48 (%)	OR (95% C.I.)	p-value
Sex				
Female	26 (41.9)	17 (35.4)	0.76 (0.35-1.65)	0.488
Male	36 (58.1)	31 (64.6)		
Age				
0-5	9 (14.5)	6 (12.5)	1	
6-15	25 (40.3)	16 (33.3)	1.04 (0.31-3.49)	0.947
>15	28 (45.2)	26 (54.2)	0.72 (0.22-2.30)	0.576
Location				
Outside MHD town	49 (79.0)	40 (83.3)	1.32 (0.50-3.52)	0.570
MHD town	13 (21.0)	8 (16.7)		
Caste				
General upper class	8 (12.9)	4 (8.3)	1	
Backward class	37 (59.7)	29 (60.4)	0.64 (0.17-2.33)	0.496
Schedule lower class	17 (27.4)	15 (31.3)	0.57 (0.14-2.27)	0.422
Previous VL treatment with SSG in family				
No	49 (80.3)	44 (91.7)	2.70 (0.81-8.97)	0.106
Yes	12 (19.7)	3 (8.3)		
Time to treatment, weeks				
0-12	48 (77.4)	39 (81.3)	1.26 (0.49-3.23)	0.625
>12	14 (22.6)	9 (18.8)		
Place of treatment				
Private	8 (12.9)	8 (16.7)	1.35 (0.47-3.90)	0.580
Government	54 (87.1)	40 (83		
Full duration of treatment with SSG (30d)				
No	33 (53.2)	14 (29.2)	0.36 (0.16-0.80)	0.012
Yes	29 (46.8)	34 (70.8)		
Mean local arsenic level (tube wells, n=5)				
<10 μg/L	45 (72.6)	39 (81.3)	1.64 (0.66-4.09)	0.291
≥10 μg/L	17 (27.4)	9 (18.7)		

Note: *This number includes 10 deaths

clinical improvement, 8 (7%) patients relapsed and 4 (4%) terminated their treatment due to toxicity. There was a high death rate in our cohort: 21 (19 %) of 110 patients had died by the date of the interview (Figure 3.13C). Sixteen (15%) of these patients died from VL (Figure 3.13D), 6 of these deaths occurred after the 6 month follow up period for SSG treatment failure so they are not included in the primary outcome. One of these deaths was directly due to treatment toxicity. Five patients died from non-VL causes including HIV (n=1), asthma (n=1), liver failure (n=1), paralysis (n=1) and a road traffic accident (n=1). Treatment outcomes were not recorded in the PHC register, PHC treatment cards or seldom in patient's hand held documents.

3.10 Arsenic exposure

3.10.1 Tube well water

The arsenic concentration in the wells tested ranged from < 3 μg L^{-1} up to 1050 μg L^{-1} arsenic. Fifty (44%) of the study subjects had at least one arsenic contaminated tube well in their home or surrounding area and 26 (24%) of them had a mean local arsenic level ≥ 10 μg L^{-1} (Table 3.7) and (Figure 3.13A). Five (4.5%) patients had a mean local arsenic level of >50 μg L^{-1}, the previous Indian Government limit. Only 11 (10%) patients were aware of the issue of arsenic contamination and 8 of these lived in an area with local arsenic contamination. The remaining 18 out of the 26 patients living with local arsenic contamination had no knowledge of the issue.

For the cut off ≥10 μg L^{-1} arsenic the areas under the ROC curves were (1) 0.54 for treatment outcome, (2) 0.69 for all-cause mortality and (3) 0.65 for VL mortality. The sum of sensitivity and specificity was greater than 100% for all outcomes. The quality control assays showed a good agreement (Kappa=90%, $p<0.001$) between the analysis performed at SOES and the University of Aberdeen laboratories.

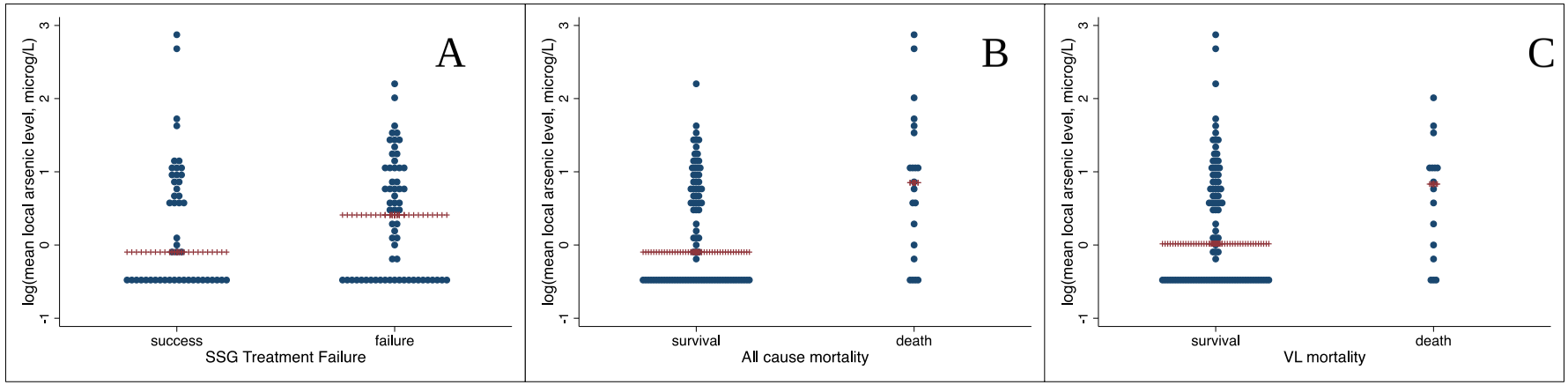


Figure 3.14 Dot plots of arsenic exposure from tube wells and SSG treatment outcome and mortality in antimonial treated cohort (n=110)

Panels A, B and C show the log of the mean arsenic level plotted against outcome: SSG failure (MWU p=0.42), all-cause mortality (MWU p = 0.005) and VL mortality (MWU p = 0.048). MWU= Mann Whitney U.

3.10.2 Urine samples

Of the 69 patients where biological samples were available, only 66 were in the final cohort of 110 and one sample was unable to be analysed, therefore arsenic exposure in urine was only able to be assessed on an individual basis in 65 (59%) study subjects.

The correlation between arsenic in urine and in the water in the study subject's local environment gives an R-squared value of 0.60 ($P < 0.0001$). From this relationship 45 urinary values were imputed to allow analysis of the full cohort. All the results for urinary arsenic analysis will be presented for 2 datasets: biological samples only (n=65) and with the imputed values (n=110).

For the cut off >40 $\mu g\ L^{-1}$ urinary arsenic the areas under the ROC curves were 0.53 and 0.49 for the two datasets respectively. The sum of sensitivity and specificity was ≥100 % for both datasets. The quality control assays showed a moderate agreement (Kappa=72%, p=0.012) between the analysis performed at SOES and the University of Aberdeen laboratories.

3.11 Treatment failure

3.11.1 Tube well water

Although the median arsenic water exposure level was higher in patients with treatment failure (Fig 3.14, panel A), this difference was not statistically significant (Mann Whitney U p = 0.42).

Only one covariate had a weak association with treatment failure ($p<0.2$) in the bivariate analysis: previous SSG treatment in the family (p=0.11)(Table 3.7). This variable was not retained in the final multivariate model for lack of significance. Although the association between treatment duration and outcome was statistically

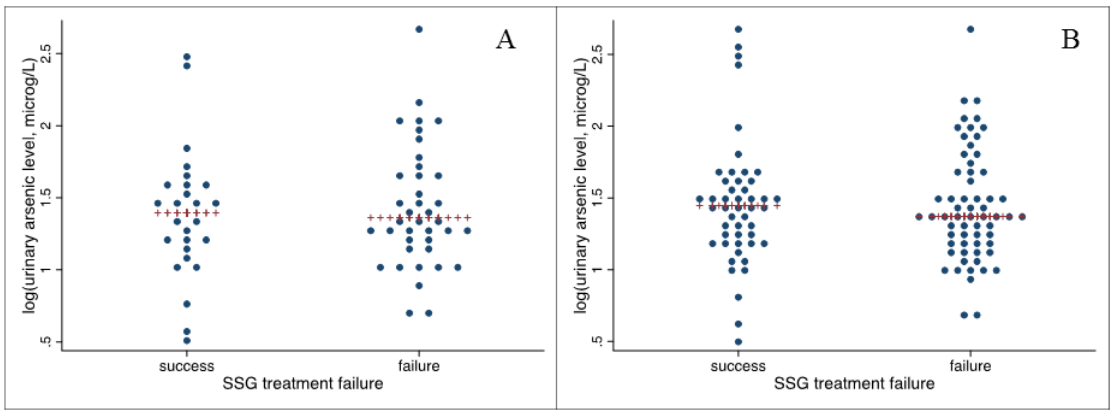


Figure 3.15 Dot plots of urinary arsenic exposure and SSG treatment outcome

Panels A and B show the log of the urinary arsenic level plotted against SSG treatment outcome: Urine biological samples (Panel A, MWU p=0.81), urine with imputed values (Panel B, MWU p = 0.80). MWU= Mann Whitney U.

Table 3.8 Multivariate logistic regression analysis using urinary arsenic data

	Urine biological samples n=65		Urine with imputed values n=110	
Variable	OR (95% C.I.)	p value	OR (95% C.I.)	p value
Urine arsenic level				
> 40 μg/L	2.06 (0.57-7.47)	0.269	1.29 (0.53-3.19)	0.575
Possible confounding factors				
Age (years)				
0-5	Ref. cat		Ref. cat	
6-15	9.57 (1.36-67.2)	0.023	1.17 (0.34-4.10)	0.798
> 15	6.95 (1.03-46.9)	0.047	0.86 (0.25-2.94)	0.808
Sex				
Female vs male	0.62 (0.20-1.96)	0.419	0.8 (0.36-1.81)	0.595
Location				
Outside of or within Mahudin Nagar town	0.97 (0.23-4.14)	0.964	1.32 (0.49-3.57)	0.582

OR = Odds Ratio, CI = Confidence Interval. Ref. cat = Reference category

significant (p=0.01) this variable was not included in the final model as in the majority of cases the treatment duration is dependent on the outcome. The final logistic regression model only included the forced variables age, sex and location and shows a trend: patients with high mean local arsenic level have a higher risk of treatment failure (OR=1.78) than patients using wells with arsenic concentration <10 μg L^{-1}, however this association is not statistically significant (95% CI: 0.7 – 4.6, p=0.23).

3.11.2 Urine samples

The median urine arsenic exposure values were similar in patients who failed and had a successful treatment with SSG (Figure 3.15, A and B). The covariate of previous SSG treatment in the family with a weak association with treatment failure (p=0.103) was initially included in a multivariate model. The final logistic regression model only included the forced variables age, sex and location. When analysing the biological samples only a trend was observed: patients with elevated urinary arsenic have a higher risk of treatment failure (OR 2.06) than those with an arsenic level within the normal range, however this association is not statistically significant (95% CI: 0.57-7.47, p=0.269). When using the data set that includes imputed values, the risk of treatment failure with elevated urinary arsenic was markedly attenuated (OR 1.29) and the association became less significant (95% CI: 0.53-3.19, p=0.575) (Table 3.8).

In the multivariate analysis using biological samples only, being of an older age carried a significant risk when adjusted for urine arsenic exposure level, location and sex. This risk disappears when the imputed data is also used in the analysis: all the patients who were not available for biological sample collection due to relocation were older than 5 years old at their time of treatment and 66.7% of this group had a successful 1st treatment with SSG (compared with a 43.6% success rate in the full cohort).

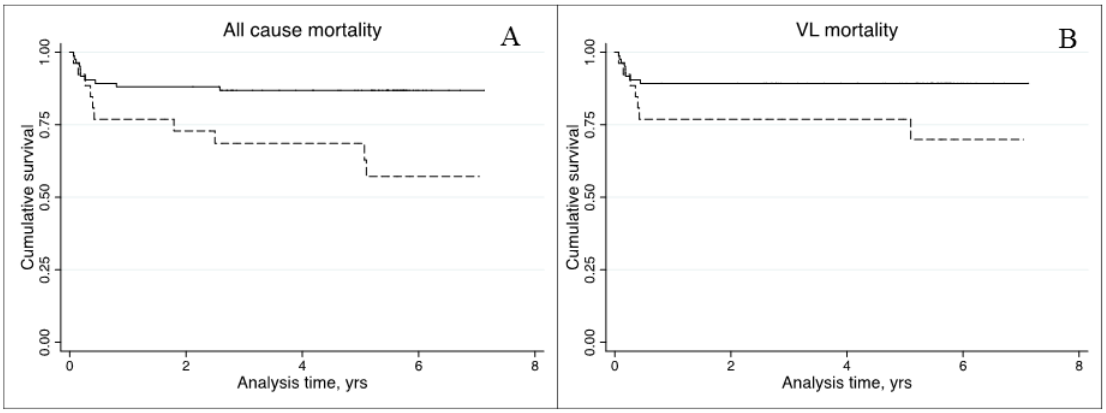


Figure 3.16 Kaplan Meier curves for all-cause and VL mortality

Panels A and B are survival curves comparing arsenic exposed (dotted line, => 10 μg L^{-1} As) and non-arsenic exposed (solid line, <10 μg L^{-1} As) patients in the outcomes of all-cause mortality (log rank p=0.004) and VL mortality (log rank p = 0.044).

Table 3.9 Multivariate Cox regression analysis of effect elevated arsenic water levels (>= 10 μg L^{-1}) on risk of the outcomes of all-cause and VL related mortality in a cohort of SSG treated patients

Variable	All-cause mortality		VL mortality	
	HR (95% CI)	p value	HR (95% CI)	p value
Effect of arsenic exposure*				
- during first 3 months from onset of VL symptoms	0.90 (0.19- 4.37)	0.896	0.92 (0.19 - 4.53)	0.921
- after 3 months from onset of VL symptoms	8.56 (2.52-29.1)	0.001	9.27 (1.75-49.0)	0.009
Possible Confounding Factors				
Age				
0-5	Ref. cat.		Ref. cat.	
5-15	0.14 (0.04-0.47)	0.001	0.11 (0.03-0.45)	0.002
>15	0.19 (0.06-0.56)	0.003	0.17 (0.05-0.56)	0.003
Sex – female vs male	1.4 (0.53-3.67)	0.498	1.22 (0.41-3.61)	0.725
Location –outside of or within Mahudin Nagar town	1.04 (0.33-3.25)	0.951	1.30 (0.40-4.28)	0.662

HR = Hazard Ratio, CI = Confidence Interval. Ref. cat = Reference category

Table 3.10 Characteristics of patients who died from VL during treatment

Patient no.	Age at time of first treatment (years)	As^{III} exposure, µg L^{-1}	Sex	Time to seek care (days)	Time to 1st treatment (days)	Treatment received (duration in days)
No arsenic exposure						
1	3	0	f	14	87	SSG (8)
2	4	0	f	2	23	SSG (5)--1-- AmpB (3)
3	4	1	m	3	21	SSG (4)
4	4	7	f	11	63	SSG (3)
5	4.5	0	m	4	16	SSG (30)--7-- SSG (15)
6	6	1	m	1	34	SSG (26)
7	40	2	f	11	17	SSG (30)--4-- SSG (15)
8	40	3	m	6	37	SSG (3)
9	50	6	m	2	55	AmpB (45)--45--SSG (8)--0-- AmpB (6)
Arsenic exposure						
10	3	40	m	8	87	SSG (10)
11	5	12	f	10	62	SSG (30)--8--SSG (30)--4-- Milt (10)
12	10	102	f	4	20	SSG (4)
13	11	33	m	10	114	SSG (16)
14	37	12	m	11	38	SSG (15)
15	45	12	m	3	94	SSG (60)
16	60	12	m	10	11	SSG (28)--1800-- SSG (5)

All patients died during treatment. --x-- represents the number of days between treatments. SSG =sodium stibogluconate, Milt = miltefosine, AmpB = amphotericin B

3.12 Mortality

As shown in the dot plots (Fig 3.14, panels B and C) the median arsenic concentrations in water are significantly higher in the patients who died (group “all-cause mortality” (p = 0.005) and those who died due to VL (group “VL mortality” p = 0.048) compared to the patients that were still alive at the time of the field visits.

The KM curves (Fig 3.16, panels A and B) confirmed increased mortality in subjects exposed to elevated arsenic water levels (p=0.004 and p=0.044 for “all-cause” and “VL mortality” respectively). Age was the only covariate to show a significant association with mortality and thus the final models only included the forced variables of age, sex and location. The multivariate Cox regression model showed that arsenic levels $\geq$ 10 $\mu g\ L^{-1}$ increased significantly the risk of all-cause (HR 3.27; 95% CI: 1.4-8.1) and VL related (HR 2.65; 95% CI: 0.96 – 7.65) mortalities. However, the effect of arsenic exposure on all-cause mortality was found to vary with time. On the KM plots, the two survival lines representing arsenic exposed and non-exposed populations separate at 3 months where the effect of arsenic exposure becomes apparent. This was confirmed by a modified Cox regression model which allowed the effect of arsenic exposure to vary over time. No increased risk of mortality was detected with arsenic exposure during the first 3 months after start of VL symptoms. However, after 3 months, the effect of arsenic exposure significantly increased the risk of all-cause mortality (HR 8.56; 95% CI: 2.5 – 29.1) and of VL related mortality (HR 9.27; 95% CI: 1.8 – 49.0)(Table 3.9). Characteristics of the individuals who died from VL are summarised in Table 3.10.

Chapter 4 - Discussion

4.1 Introduction

The development of drug resistance in pathogens is a dreaded complication in any infectious disease. This is particularly true in diseases without an established vaccine. Antibiotic resistance is a topical issue in the UK currently with David Cameron warning that "medicine could soon be cast back into the dark ages" if infections that were previously treatable become incurable due to multi-resistant pathogens[17]. In response the UK government has recently established a working committee to try and reduce antibiotic resistance, develop new antibiotics and reduce un-necessary antibiotic use.

This current situation in the UK has parallels with the situation in Bihar in the mid-1990s when antimonial treatment failure rates for visceral leishmaniasis fell to around 50 % and resistance in the *Leishmania* parasite was demonstrated. The progress in developing effective pentavalent antimonial compounds made by Bramachari and Leonard Goodwin (Section 1.2.2) had been undone and new drugs were urgently needed to treat an otherwise fatal disease. Some of the measures currently outlined for the UK came to pass in India with the development of amphotericin B, miltefosine and paromomycin as alternative treatments (Section 1.1.7) and the improvement in diagnostics, and thus reduction of misprescribing, with the advent of the rK39 rapid antigen test (Chappuis *et al.*, 2006)(Section 1.1.6). Why antimonial resistance had developed so rapidly in India when antimonial compounds remained remarkably effective in the treatment of VL in Africa and South America was not fully addressed. Current theories include the unrestricted use in India through private prescriptions and over the counter procurement.

[17] http://www.bbc.co.uk/news/health-28098838

The development of multi-resistant pathogens in the UK is being blamed on overprescribing, the unregulated use of antibiotics in countries such as China and India (Van Boeckel *et al*., 2014) and the heavy use of antibiotics in livestock (Alcorn, 2012). In the study of resistant micro-organisms a clear picture emerges – the more antibiotic use, the more resistance (Albrich *et al*., 2004; Malhotra-Kumar *et al*., 2007). With less antibiotic use, resistance decreases (Monnet, 2000). However with antimonial resistance in *Leishmania*, not least as it is not part of natural carriage in humans, the situation is more complicated. There are differing reservoirs between countries, some with antimonial exposure, e.g. treatment of dogs in Spain and South America. There are differing levels of unregulated treatment access between affected countries – a vial of antimony can be bought with a bull in Sudan (personal communication Margriet Den Boer) – making useful epidemiological data collection difficult. In the Indian subcontinent, although antimonial use has decreased since it was no longer recommended for routine use, both high levels of treatment failure and resistance remain. Other factors must be in play which may be related to host susceptibility, the parasite itself or the environment – for example exposure to arsenic, the topic of this thesis.

It is important to look into the ecosystem of an organism to establish the reasons for resistance development (Ait-Oudhia *et al*., 2011). In looking further than human antibiotic use, one hypothesis being investigated is how the livestock industry's antibiotic practices, particularly the use of growth promoting antibiotics in animal feed, are leading to human drug resistant pathogens. It has been difficult to strongly establish the links, as there are many aspects to the food chain. However, in 2012 whole genome analysis has demonstrated that the CC398 life stock associated MRSA was originally MSSA in humans and acquired methicillin and tetracycline resistance from antibiotics in animal feed after it was introduced to livestock from humans (Price *et al*., 2012).

Although this is just one strain of one bacteria it demonstrates the potential of the sub-therapeutic use of antibiotics in animals can lead to drug resistant pathogens in humans and the importance of looking at the whole picture.

This work looks at the ecosystem of *Leishmania* in the Indian subcontinent and has aimed to look at the study of *Leishmania* resistance and *Leishmania* resistance selection at different levels. It asks whether sub-therapeutic exposure of *Leishmania* to arsenic within the exposed population could have contributed to the development of antimonial resistant parasites and the high rates of treatment failure observed. The key findings are summarised below:

- The method of resistance selection has an effect on speed of resistance development and resistance phenotypes of *Leishmania* life cycle. This has important implications for laboratory study of resistance.
- Cross resistance between the metalloids arsenic and antimony can be selected for in axenic amastigotes, but this resistance is accompanied by phenotypic changes that diminish the value of the generated resistant line for mechanistic studies.
- Resistance that was selected for, *in vitro* or *in vivo,* intra-macrophage in the amastigote form, leads to amastigote stage-specific resistance which may be related to antimony reductase and is not solely thiol dependent.
- Oral arsenic exposure of mice at environmentally relevant levels can induce cross resistance to antimony that is relevant *in vivo* and *in vitro*.
- A strong association between arsenic exposure and antimonial treatment failure was not detected in a field setting.

- There was an unexpectedly high mortality rate in the cohort of SSG treated patients and a secondary analysis of mortality showed that arsenic exposure strongly increased the risk of both all-cause mortality and VL mortality.
- Arsenic exposure strongly increases the risk of death from VL particularly if there is a delay to treatment.

4.2 Selection of antimonial resistance

Antimonial resistance has been selected for at three levels in this work, in axenic amastigotes, in intra-macrophage amastigotes and an *in vivo* model. Each method has revealed interesting findings relating to the study of antimonial resistance.

4.2.1 Antimonial resistance selection in axenic amastigotes

Axenic amastigotes were developed as a tool to study the amastigote form without the complication of the macrophage. As previously described by Ouellette it is possible to generate axenic amastigotes cross resistant to antimony and arsenic through progressive exposure to increasing concentrations of trivalent antimony (Brochu *et al*., 2003; El Fadili *et al*., 2009; El Fadili *et al*., 2005). However, in the current study, we found that maintaining the *Leishmania* parasite in axenic form for prolonged duration led to an unstable phenotype, with promastigote features despite growing in amastigote culture conditions. The tolerance developed to the metalloids at high concentrations was not maintained through the cloning process for the lines exposed to Sb species and the arsenic resistant line required prolonged passage at high arsenic concentrations for the metalloid resistance to be retained. Of note Ouellette's group did not clone their Sb resistant generated parasites. Additionally no documentation is made of the phenotypic appearance or the duration of exposure prior to removal of the drug for 20 passages to create partial revertants. In my work prolonged exposed of > 150 days was required for selection of stable resistance.

Although extensively studied in *L.tarentolae* promastigotes, arsenic has not previously been used to select for resistance in axenic amastigotes. In my study, it would have been useful to compare the arsenic and antimonial - selected resistant lines to identify any key mechanistic differences to help to answer the question of how antimony resistance had become so prevalent in Bihar. Unfortunately, the phenotypic changes, the low baseline susceptibility to antimony naturally present in the clone LdSu3 and the decreased virulence of LdBob prevented *in vivo* work with these strains and significantly devalued the use of axenic amastigotes for any kind of comparative work. Additionally it would have been ideal to be expose multiple lines of the same original clone to the different metalloids to allow for more robust comparison of any mechanistic changes identified. Dramatic differences in resistance mechanisms can be found in strains from clones selected using the same resistance method (Lin *et al*., 2008).

The development of axenic amastigotes as a biological tool was initially heralded with great excitement within the parasitological world as it would facilitate study of amastigote function (Debrabant *et al*., 2004), resistance development (El Fadili *et al*., 2009; El Fadili *et al*., 2005; Goyard *et al*., 2003) and had potential use for drug screening programmes (Callahan *et al*., 1997). Axenic lines were found to be able to infect animals and have many similarities to intracellular amastigotes including acid phosphatase production and the presence of the A2 marker (Debrabant *et al*., 2004). However more recent work has identified significant differences between axenic and intracellular amastigotes including changes in the key enzyme tryparedoxin peroxidase and in enzymes involved in protein and amino acid metabolism (Pescher *et al*., 2011). Keith Gull's group performed RNA sequencing on *L.mexicana* and found that while 1665 annotated genes were differentially expressed between promastigotes and amastigotes, axenic amastigotes appeared to be an intermediate phenotype with 143

genes differentially expressed between intracellular amastigotes and axenic amastigotes and 576 between axenic amastigotes and promastigotes[18]. Another RNA study comparing axenic amastigotes and intracellular amastigotes showed that the groups only shared 12% of differentially upregulated genes (Rochette *et al*., 2009). A protein screen comparing antimonial sensitive and resistant axenic amastigotes should be interpreted in light of the recent findings with axenic amastigotes (El Fadili *et al*., 2009; Pescher *et al*., 2011; Rochette *et al*., 2009)[2]. Additionally drug screening programmes have found a high false positive hit rate with axenic screening assays versus intracellular assays (De Rycker *et al*., 2013).

An interesting question relating to the axenic amastigote model is -what is the relevance of the late log axenic amastigotes in a culture flask when the natural environment of an amastigote is resident in a macrophage? The differences in sensitivity to arsenic between mid-log and stationary phase amastigotes observed is likely related to depletion of nutrients. Do amastigotes reach this point within macrophages in the body and is this the trigger for amastigotes to escape and infect new macrophages? It is not known whether this disruption occurs prior to a 'stationary phase' within the macrophage being reached. Macrophages within tissue have the ability to live for many months (Gonzalez-Mejia and Doseff, 2009) – so it is possible that a saturation point of amastigotes within a macrophage could be reached. This may have implications on the sensitivity to arsenic or antimony in a patient with very heavily parasitized organs.

The use of any laboratory derived strain is a compromise. Laboratory strains lose their *in vivo* virulence with as little as 20 passages (Ali *et al*., 2013). This work has

[18] Eva Gluenz, Michael Fiebig, Steve Kelly and Keith Gull.
Insights into the missing biology of *Leishmania Mexicana* from RNA sequencing.
Abstract A101. British Society of Parasitology 52nd Annual Spring Meeting. April 2014

demonstrated that even the correct laboratory handling of LdBob significantly changed its sensitivity to Sb^V in the amastigote form. Although the use of laboratory strains is helpful as they can be well characterised and their handling history is known, confirmation of any findings within clinical isolates is imperative.

4.2.2 Selecting for resistance in macrophage through Sb^V exposure

To evaluate resistance selection in the intracellular amastigote rather than the axenic amastigote an intracellular macrophage method was used (Hendrickx *et al*., 2012). This approach generated 10-fold resistance rapidly in LV9 amastigotes with only 3 passages of 5 day exposures to Sb^V. The resulting drug-exposed parasites displayed an amastigote stage-specific resistance to antimony up to levels of 4.1 mM with no difference found in promastigote susceptibility to trivalent antimony.

This stage-specific susceptibility may be related to loss of the activation step of Sb^V to Sb^{III} via one of the putative reductases TDR1 or LmACR2 or an as yet unidentified reductase. Further work will include the analysis of the genomic sequence of the 6 lines A-F to look for specific differences between the sensitive and resistant isolates particularly in reference to antimony reductases. Another possible explanation for this stage-specific resistance would be loss of Sb^V transport into the cell.

The speed of resistance development in the macrophage was interesting. In total 15 day of drug exposure is much shorter than the 108 days required using axenic amastigotes to induce 10 fold resistance. It is also half the length of the recommended 30 day treatment course implying that sub-therapeutic antimony in dose or duration could easily lead to resistance.

This method has previously been used in antimony-resistant clinical isolates to select resistance to paromomycin and miltefosine (Hendrickx *et al*., 2014; Hendrickx *et al*., 2012) but not to induce resistance to antimony. Paromomycin-resistant isolates were

developed rapidly in just two 5–day cycles and displayed a similar pattern of amastigote stage-specific resistance. Conversely, miltefosine did not demonstrate a difference in intracellular amastigote EC_{50} but over 7 successive passages backtransformation of promastigotes occurred at higher concentrations of miltefosine (Hendrickx *et al*., 2014). These backtransformed promastigotes however did not display resistance to miltefosine in an axenic EC_{50} assay. These findings highlight the importance of the method of resistance selection and that both phenotypes should be tested in assessing parasite susceptibility to a given compound.

It is important to remember though that the type of macrophage used may have an effect on results. This was evident in the different amastigote infection levels that we observed with between peritoneal derived mouse macrophages and THP-1 cells in our study. Another group demonstrated up to a 3- fold difference in the EC_{50} between THP-1 and peritoneal derived mouse macrophages and when using human peripheral blood monocytes a 10 fold decrease in Sb^V EC_{50} was observed when compared to peritoneal derived mouse macrophages (Seifert *et al*., 2010).

4.2.3 Leishmania Sb^V resistance selected for by environmental arsenic exposure

The *in vivo* work in this study demonstrates that it is biochemically and physiologically plausible that arsenic exposure in the environment could have contributed to the development of antimonial resistance in *Leishmania* in Bihar (Perry *et al*., 2013).

As discussed previously, arsenic has long been known to generate cross resistance to antimony although this has not previously been demonstrated in an *in vivo* model. The speed of resistance development was positively correlated to the level of exposure: at the level of hepatic arsenic of 0.4 to 1.5 mg kg^{-1} seen in the 100 ppm group, the exposed parasites had become resistant to 4.1 mM Sb^V *in vitro* within 3 months. At the lower

exposure level of 10 ppm, which is equivalent to a 60 kg man drinking 3L of 1200 µg L^{-1}, resistance at this level took up to 5 months to develop.

This work was performed using the WHO reference strain LV9 originally obtained from an Ethiopian patient in 1967. The line used had been passaged through hamsters since 2002 and through BALB/c mice for one year before use in the arsenic model. It is difficult to say how this handling history has affected our results including the speed at which the LV9 parasites gained resistance. Ideally, it would be interesting to repeat this resistance selection using clinical isolates from Bihar to see whether the parasites from the area in which arsenic exposure occurs behave in the same way as LV9.

4.2.3.1 Arsenic exposure in Bihar

This level is in the upper bracket of arsenic contamination in tube wells detected so far in Bihar. Only 2.1% of all the wells tested by SOES[19] had levels greater than 500 µg L^{-1}. There have been 2 main surveys performed in Bihar: UNICEF who surveyed 66,623 wells, 28.9% were > 10 µg L^{-1} and 10.8% > 50 µg L^{-1} (Nickson *et al*., 2007); and SOES who measured 19,961 wells, 32.7% were > 10 µg L^{-1} and 17.8% > 50 µg L^{-1}. Neither of the surveys were exhaustive and there was potential inaccuracies in the UNICEF field kits and sampling bias in the SOES study towards villages where patients were suspected (Ravenscroft *et al*., 2009). However, the geographical bias towards areas within 10 km of the Ganges River (Nickson *et al*., 2007) is the main concern and more widespread testing is required.

4.2.3.2 Arsenic exposure model

The relevance of this hypothesis to Bihar is defined by how closely our model relates to the development of antimonial resistance in the leishmania parasite through arsenic exposure in patients. The model of arsenic exposure that we used employed high

[19] http://www.soesju.org/arsenic/arsenicContents.htm?f=groundwater_bihar.htm

concentrations of arsenic to recreate in weeks hepatic arsenic levels that will have built up over years in arsenic exposed populations. This was confirmed by ICP-MS measurements. There have been criticisms of utilising the Reagan Shaw body surface area dose conversion equation (Reagan-Shaw *et al*., 2008; States *et al*., 2011)(Section 2.10) between humans and mice as mice methylate arsenic more efficiently than humans (Vahter, 1999). Additionally, there has been a call to use phenotypic anchors such as arsenic levels in body tissues as we have done by matching hepatic arsenic levels (States *et al*., 2011)

Furthermore, as methylation helps to eliminate arsenic from the body and mice are efficient methylators (Vahter, 1999), using the high levels of arsenic in murine drinking water may have helped to ensure hepatic levels were reached. Although we did not perform arsenic speciation, it has been performed on pregnant C3H mice exposed to 85 ppm arsenic for 10 d. A hepatic level of total arsenic of 1.6 mg (kg wet weight)$^{-1}$ was obtained, of which 37.6% ± 10.4 was inorganic As, 18.9% ± 2.9 MMA species and 43.6 ± 13.2 % DMA species (Devesa *et al*., 2006).

In another study where C3H mice were orally dosed with high doses of 50, 100 and 150 µg As day^{-1}, 6 days a week for a year, hepatic arsenic levels continued to rise (Das *et al*., 2005). At 10 ppm exposure in drinking water, where our BALB/c mice where consuming on average 20 µg day^{-1}, reaching hepatic arsenic levels of 0.12 ± 0.02 mg (kg wet weight)$^{-1}$ at 56 d, Sb^{V} resistance was selected for following 5 months of exposure. Over time it is possible that even at lower levels of exposure this level of hepatic arsenic levels would be reached in mice. Furthermore, the incubation period of VL is weeks to months and it is possible that with a longer exposure period resistance could be selected for in the leishmania parasite at lower hepatic arsenic levels.

It is worth noting that recent work has shown that the gut microbiome affects both methylation and thiolation of ingested arsenic (Lu *et al*., 2014; Rubin SS *et al*., 2014) which will in turn affect the concentrations of arsenic in internal organs. The gut microbiome is affected by host genetics (Lu *et al*., 2014) and diet. This is another important difference between the mouse model and chronic exposure to arsenic in humans.

4.2.3.3 In vivo assessment of arsenic generated antimonial resistance

The assessment of *Leishmania* Sb^V resistance in the in macrophage assay is an imperfect tool for predicting clinical failure with antimonial treatment (Rijal *et al*., 2007). One reason for this may be related to the dual action of Sb^V because, as well as the toxic effects of Sb^{III}, Sb^V itself stimulates the production of reactive oxygen species and nitrosative stress in mice via the stimulation of production of IL-12 and IFN-γ (Murray *et al*., 2000). Importantly, the results of the *in vivo* treatment with Sb^V show that the resistance developed in the arsenic exposed *Leishmania* is relevant in the *in vivo* setting of our arsenic exposure model. Additionally it showed that this resistance did not affect response to miltefosine treatment which is relevant to the VL elimination programme.

The mice continued to be exposed to arsenic during the antimony treatment. The reported effects of arsenic on the human immune system include decreased expression of inflammatory genes (Andrew *et al*., 2008), depressed macrophage function and nitric oxide production (Banerjee *et al*., 2009) and decreased production of inflammatory cytokines including IFN-γ (Biswas *et al*., 2008). If antimony treatment cannot overcome this immunosuppressive state then the immune arm of its function would be disabled, which, along with the demonstrated antimonial resistance, could have contributed to the treatment failure of antimony in this *in vivo* model.

4.2.3.4 Hepatic concentrations of arsenic and antimony

An intracellular concentration of hepatic arsenic and antimony was calculated, taking the hepatocytes per gram of liver to be 135 x 10^6 (Sohlenius-Sternbeck, 2006) and the volume of a hepatocyte to be 3.4 x 10^{-9} ml (Alberts *et al*., 1983). It is not known whether hepatocytes and Kupffer cells take up arsenic at the same rate but that has been assumed in this calculation. In the mice exposed to arsenic at 10 and 100 ppm the concentration of arsenic in hepatocytes is estimated to be 3.5 µM and 11.8 µM, respectively and the corresponding antimony concentration 72h post treatment is estimated to be 35.8 µM. The arsenic concentration represents a near steady state whereas the antimony concentration at 72 hours post treatment reflects an approximate 10-fold decrease from 1 hour post treatment, with an initial rapid decline in the first 8 hours, followed by a slow elimination phase (Berman *et al*., 1988). This decrease correlates well with an in macrophage EC_{50} of 316 µM (36.5 µg ml^{-1}) at the point of treatment.

The real question is what is the concentration in the phagolysosome where the parasite resides? It is possible that an average concentration over all the cells in the liver is an underestimate of the concentrations the parasites experience in the Kupffer cell. An ultrastructural study of infected macrophages incubated with sodium stibogluconate suggests that Sb^V concentrates in the phagolysosomes (Abok *et al*., 1988). This built on work showing delivery of antimony into the phagolysosomes by liposomes (Heath *et al*., 1984). Due to the similarities between arsenic and antimony it is possible that with arsenic also accumulates in the secondary lysosomes of the macrophage and thus the calculated concentrations are lower than the concentrations the amastigotes were exposed to in this *in vivo* model.

There was no difference seen in hepatic antimony levels between non-arsenic exposed and arsenic exposed groups implying that the presence of arsenic did not interfere with the metabolism of Sb^{V} and that altered antimony metabolism was not responsible for the decreased efficacy of Sb^{V} in the arsenic-exposed groups. This observation is confirmed by uptake studies where the presence of As^{III} did not interfere with the uptake of Sb^{V} (Brochu *et al*., 2003).

4.2.3.5 Effect of arsenic exposure on parasite growth

The parasite burden in the 3 groups over the 5 passages shows that in the first passages the parasite load in the 10 ppm, but particularly in the 100 ppm group was depressed. This may be due to a treatment effect of the arsenic which was overcome as the arsenic-exposed parasites became resistant. This is a trend and not statistically significant. However this trend raises the question of whether the arsenic accumulation, in a heavily exposed patient, could be protective against clinical infection with a sensitive strain of *Leishmania*.

In the 10 ppm the parasite load at the 5th passage has risen to greater levels than the control group, although, again, this is not statistically significant. This rise could be interpreted in two ways: first, that the resistant parasites are fitter and stronger as previously demonstrated by Jean Claude Dujardin's group (Vanaerschot *et al*., 2011) or secondly that the dampening effect of arsenic on the immune system (Dangleben *et al*., 2013) led to an increased *in vivo* parasite burden now the parasites were not experiencing a treatment effect.

4.2.3.6 Analysis of in vivo generated resistance

When tested for their susceptibility to the trivalent metalloids Sb^{III} and As^{III}, it was surprising that promastigotes derived from the arsenic exposed groups of mice (10 ppm and 100 ppm) remained as susceptible as the baseline group (0 ppm). It must be taken

into account that the promastigotes tested were passaged through non-exposed mice for a total of 8 months, due to issues finding the right conditions for promastigote differentiation, prior to susceptibility testing. Therefore these parasites may be partial revertants.

However, when the promastigotes were used to infect macrophages, the amastigote forms of the exposed groups, 10 and 100 ppm, remained resistant to up to 4.1 mM Sb^{V}. This stage-specific resistance, together with the similarity in thiol levels between all 3 groups (Table 3.5) allows for speculation that the mechanism of antimonial resistance generated is either through loss of the ability of Sb^{V} to enter the parasite or through loss of the activation step of Sb^{V} to Sb^{III} within the leishmania parasite.

Although the exposure of the mice was to trivalent arsenic, this species is rapidly metabolised in the liver to MMA^{V} which is reduced to MMA^{III}. In view of the sensitivity of the promastigotes to As^{III} we hypothesised that the loss of a pentavalent reductase may be responsible for the resistance of the 10 and 100 ppm arsenic exposed parasites and went on to determine the susceptibility of the 0, 10 and 100 ppm parasite groups to MMA^{V}. The 10 and 100 ppm intracellular amastigotes were insensitive to MMA^{V} at levels up to 1.5 mM compared to the 0 ppm group which had an EC_{50} of 0.38 mM implying that the enzyme required to activate MMA^{V} to the more toxic MMA^{III} was absent or reduced in the arsenic exposed groups. Interestingly, wild type LV9 promastigotes, which have an EC_{50} for As^{V} of 0.05 mM and are thought to express TDR1 at significantly lower levels than amastigotes (Denton *et al*., 2004), were refractory to MMA^{V} at concentrations up to 15.4 mM. It is worth noting though that the TriTrypDB database[20] shows that RNA levels are in fact1.4 fold down-regulated in amastigotes versus promastigotes. However this data is less robust as it is well

[20] http://www.tritrypdb.org/tritrypdb/

established that the levels of RNA are not necessarily a reflection of the levels of protein in *Leishmania* (Rosenzweig *et al*., 2008).

The putative *Leishmania* antimony reductase TDR1 has a reported specific activity for MMA^V of 18.6 ± 6.9 μM min^{-1} (mg of protein)$^{-1}$ (Denton *et al*., 2004) which is in fact greater than its activity for sodium stibogluconate of 6.3 ± 2.4 μM min^{-1} (mg of protein)$^{-1}$. The enzyme is similar to glutathione-S transferase omega which is the enzyme thought to be responsible for MMA^V reduction in humans (Xu *et al*., 2009). Unfortunately the antibody against TDR1 used to try to detect a difference in TDR1 levels between our arsenic exposed and non-exposed groups did not detect the presence of TDR1 in any group. This may be because the original experiment performed to identify the presence of TDR1 in amastigotes performed by (Denton *et al*., 2004) used a thiol-enriched lysate. The cell numbers that were available to us from our *in vivo* passage were not large enough to perform the Western Blot using a thiol-enriched lysate. Further work will include generating our own anti-TDR1 antibody. It is not known whether the other putative reductase LmACR2 can reduce MMA^V.

One important question is why was no resistance built up to the trivalent metalloids in promastigotes if the proportion of MMA species (18.9%) previously detected in the livers of mice exposed to 85 ppm, with 37.6% inorganic As (iAs) (Devesa *et al*., 2006), is similar to the proportions found in the macrophage/amastigote? One answer may be that resistance to the trivalent metalloids was present, but was subsequently lost in the long cycles without drug exposure. Another answer may be that the iAs measured is protein bound and not pharmacologically active.

It is not known whether *Leishmania* have the capacity to methylate arsenic. A BLAST search[21] (performed by Alan Fairlamb), for an equivalent enzyme to the AS3MT in humans (Section 1.3.2) (Hu and Colman, 1995) detected a number of methyl

[21] http://blast.ncbi.nlm.nih.gov/Blast.cgi

transferases, including a number of hypothetical proteins with low identity (<21%) and low E values (<$2e^{-24}$). Only one protein had an e-value <e^{-15}, the generally accepted cut-off for significance. This was 3 –dimethyl ubiquinone 9 3-methyltransferase, putative (CoQ3) involved in the biosynthesis of obiquinine. A reciprocal Blast search against the human genome identified CoQ3 as the top hit. If the leishmania parasite can methylate arsenic it is likely to be with a different enzyme to AS3MT.

In the mammalian cell MMA^{III} forms an adduct with 2 glutathione molecules and can be transported by effluxed via MRP1 in a similar detoxification mechanism as Sb^{III} with trypanothione in *Leishmania* (Carew *et al*., 2011). Antimony itself is known to be methylated to a lesser extent than arsenic and although antimony methylated species have been found in the environment (Filella *et al*., 2002) methylation is not thought to be part of the detoxification in humans (Gebel, 1997).

The transporter for pentavalent antimony to enter the amastigote is unknown. It is established to be a different uptake transporter to As^{III} which is an aquaglyceroporin. The metabolite MMA^{V} is likely to be able to diffuse into the cell and may also be able to enter the phosphate transporters used by As^{V}. Although Sb^{V} can form a similar molecule to phosphate in aqueous solution and therefore could also enter by the phosphate transporters, loss or the reduction of the phosphate transporter is unlikely to be a resistance mechanism due to the essentiality of phosphate for the cell.

4.2.3.7 Stability of resistant strains

The stability of resistance is important to this hypothesis as stability allows for spread of resistant parasites to areas where there is no arsenic contamination. There are areas of Bihar, for example Muzaffarpur, where no significant arsenic contamination has been detected (although extensive testing has not yet been performed) and high levels of antimonial resistance are reported (Sundar *et al*., 2000).

The stability of the 10 and 100 ppm amastigote resistance to Sb^V over 8 months passage *in vivo* with no arsenic exposure, and 3 months grown as promastigotes *in vitro* implies that the resistance mechanisms acquired are likely to have occurred on a genetic rather than an epigenetic level. Mutations occur frequently and as the lines 0, 10 and 100 ppm were not generated in triplicate, sequencing would not be able to correctly identify relevant single nucleotide polymorphisms.

This stability and an increased fitness has been demonstrated in clinical isolates with resistant clinical isolates generating a higher *in vivo* parasite burden, a higher proportion of metacyclic promastigotes in stationary phase and more efficient infection of macrophages (although this advantage was lost by 96 h post infection) (Vanaerschot *et al.*, 2010)(Vanaerschot *et al*., 2011). This fitness work has only been performed handling promastigotes *in vitro* and needs to be confirmed using sand fly colonies. In my study, an increased parasite burden was potentially visible in the 10 and 100 ppm parasites at the end of the fifth passage as discussed above (Section 4.2.3.4). We did not see any difference in infection levels between the three groups 0, 10 and 100 ppm but our slides were only read at 72 h so any difference may not be visible by this time point.

Amastigotes harvested from BALB/c mice were in general less efficient at entering the macrophage and required a different medium of supplemented RPMI rather than Grace's medium to differentiate as compared to ex vivo amastigotes from the Syrian hamster. This implies that although BALB/c mice do function as an animal reservoir for *Leishmania donovani* the harvested parasites have undergone changes within the BALB/c mice and appear less fit than those passaged through the hamster.

4.3 Pharmacokinetics of antimony related to resistance

The proposed pharmacokinetics of antimony proffer some interesting questions in relation to antimonial resistance. Following injection, Sb^{V} follows a 2 compartment model with a rapid elimination phase of half-life 2 h and a slow terminal elimination phase with a half-life of 76 h (Chulay *et al*., 1988). A recent study where rhesus monkeys received a 21 day course of meglumine antimonite showed a significant accumulation over the treatment course of antimony in the liver, spleen and bone marrow. Speciation of plasma showed it was the Sb^{III} that was being excreted in the longer terminal elimination phase, indicated that the conversion of Sb^{V} to Sb^{III} occurs in the peripheral organs (Friedrich *et al*., 2012).

The ability of organs to convert Sb^{V} to Sb^{III}, as suggested in this study (Friedrich *et al*., 2012), could contradict our argument that Sb^{V} is reduced to Sb^{III} within the amastigote rather than the macrophage. However it is possible that the hepatocytes, as the major metabolising cells of the body, have more of a facility to reduce pentavalent antimony than macrophages.

This marked accumulation in the liver and spleen that reached similar levels to the peak blood levels during treatment of the rhesus monkeys (Friedrich *et al*., 2012) is likely to be protective against the development of resistance as it is heterogeneity of drug concentration within a 2 compartment model that facilitates the evolution of drug resistance (Kepler and Perelson, 1998). The probable importance of the accumulation in liver, spleen and bone marrow does indicate, as concluded in 4.2.2, that shorter prescribed or unfinished courses would be very risky for the development of resistance.

The results are interesting if one applies the theory behind the “sanctuary” 2-compartment model (Kepler and Perelson, 1998) to the results obtained from the BALB/c arsenic exposure model used in this work. Without the large peak of an

applied dose of drug, the constant ingestion of low level arsenic in drinking water creates a heterogeneity between blood and plasma levels and the organs of liver and spleen, creating an ideal environment for resistance to develop in a step-wise fashion.

4.4 The clinical relevance of stage-specific resistance

In both *in vitro* and *in vivo* methods of inducing antimonial resistance used in this work a stage specific resistance was obtained in the resulting parasite where the promastigote form remained sensitive to trivalent metalloids.

Stage-specific resistance to antimony has been observed in clinical isolates from both VL and CL patients and attempts have been made to correlate this with clinical outcome in 3 studies. Observations that patients can have a successful outcome with antimony treatment but their clinical isolates are resistant to Sb^V in macrophage assays have led to a questioning of the validity of the macrophage assay as a drug susceptibility screen. Clinical isolates of *L.donovani*, *L.guyanensis* and *L.braziliensis* were tested for their susceptibility to both Sb^V and Sb^{III} and assessed for resistance using an activity index in relation to a sensitive reference strain. Out of 16 isolates, the two isolates that were resistant to both Sb^V and Sb^{III} (R/R) had a clinical outcome of treatment failure whereas the patients from which 4 isolates that were resistant to SbV but sensitive to Sb^{III} (R/S) experienced definitive cure (da Luz *et al*., 2009). In another study in Peru on CL, out of 10 isolates with Sb^V resistance and Sb^{III} sensitivity (R/S) 7 patients were cured and 3 experienced treatment failure. No statistical correlation between Sb^{III} and clinical outcome could be drawn (Yardley *et al*., 2006). Rijal in Nepal found that out of 11 isolates from VL patients with paired Sb^V and Sb^{III} data, stage-specific resistance was present but there was no statistical correlation with clinical outcome on this small sample size (Rijal *et al*., 2007). To further explore the hypothesis that Sb^V resistance with Sb^{III} sensitivity could be an intermediary phase of resistance further studies on

larger numbers of clinical isolates are required together with mechanistic studies as the above studies do not provide any concrete evidence (Maes *et al*., 2013). However gathering paired data on sensitivity to Sb^{V} and Sb^{III} is now recommended.

In the context of the above stage-specific resistance hypothesis it would be interesting to extend the exposure of the Sb^{V} exposed lines A-C to determine whether promastigotes would eventually develop resistance to Sb^{III}. If the resistance mechanism in lines A-C is the loss of the activation of Sb^{V} to Sb^{III} then, if Sb^{V} reduction occurs solely in the amastigote, the parasite would not be required to develop Sb^{III} resistance mechanisms. However if Sb^{V} reduction also occurs in the macrophage then resistance to Sb^{III} could develop.

Why patients with sole Sb^{V} resistance in macrophage (R/S) are able to experience clinical cure may be related to the action of Sb^{V} on the arm of Sb^{V} that stimulates the immune system. If the mechanism of R/S resistance is loss of the activation step to Sb^{III}- could it be that R/S patients fail if they have a suppressed immune system whereas with a fairly intact immune system, i.e. not too far into their VL disease and with no co-morbidities, the immunostimulatory effect of Sb^{V} is enough to kill the parasite without the toxic effect of Sb^{III}. It would make sense drawing from the studies of trivalent metalloid generated resistance that R/R resistant parasites are more likely to have built up the resistance mechanisms and changes in the thiol system of the amastigote that would protect against an activated immune system than R/S parasites.

4.4 Antimonial resistance mechanisms, arsenic and clinical isolates

The antimonial resistance mechanisms depicted in Figure 1.12 have all been demonstrated in *Leishmania* parasites selected for resistance to As^{III}: downregulation of

AQP1 (Lin *et al*., 2008), upregulation of MRPA (Dey *et al*., 1996), upregulation of tryparedoxin peroxidase (Lin *et al*., 2005) and the enzymes of trypanothione biosynthesis, ODC (Haimeur *et al*., 1999) and γ-GCS (Grondin *et al*., 1997) with the resulting elevation in intracellular thiols (Mukhopadhyay *et al*., 1996). Subsequently, these same mechanisms have been established in *Leishmania* selected for resistance to Sb^{III} (Al Olayan *et al*., 2002; Brotherton *et al*., 2013; Haimeur *et al*., 2000; Wyllie *et al*., 2008). This is added evidence of the similarity between arsenic and antimony as, from published work, there is no particular resistance mechanism that is unique to one or other metalloid. It is unfortunate that the selection of resistance in axenic amastigotes performed in this work did not provide samples of a quality that would allow a direct comparison to be made between resistance mechanisms to be made.

In the attempt to ask the question of whether arsenic exposure has helped to fuel antimonial resistance in the Indian Subcontinent we run into the same difficulty. What is interesting though is that the resistance patterns demonstrated in clinical isolates from this region are heterogenous and although all of the above resistance mechanisms have been demonstrated there is not one marker that is present in all isolates (Mandal *et al*., 2007; Mandal *et al*., 2010; Mukherjee *et al*., 2007; Rai *et al*., 2013b; Wyllie *et al*., 2010). One important caveat is the heterogeneity of macrophage models used to assess resistance, so one isolate could be classified as sensitive in one laboratory and as resistant in another. Assay standardisation may be a solution to this problem (Maes *et al*., 2013) including the use of an activity index based on reference strains in all clinical isolate susceptibility testing (Yardley *et al*., 2006).

It is worth discussing the relevance of the trivalent uptake transporter AQP1 in antimonial resistance if, as hypothesised in Section 1.2.4.1 and Section 4.2.3.5, the reduction of Sb^{V} takes place in the leishmania parasite. Once MRPA has effluxed the

reduced Sb^{III} thiol conjugate from the parasite into the phagolysosome dissociation could occur in the acidic oxidising conditions. This could results in the release of Sb^{III} to become available to be pumped into the parasite again via AQP1. Hence the detection of its downregulation in some antimonial resistant clinical isolates.

One would hypothesise that once a drug-resistant strain emerged through mutation it would spread clonally through anthropogenic transmission. However the range of resistance mechanisms identified in clinical isolates contradicts this. A genotyping study of Nepalese isolates confirmed polyclonal populations of resistant and sensitive parasites, demonstrated acquired resistance occurring within a patient and hypothesised a pleiotropic method of resistance development (Decuypere *et al*., 2005). This results of this work were confirmed in a latter study that looked at resistant parasites at 5 levels: DNA sequence, gene expression, protein expression, thiols and stress tests. Two distinct populations were analysed and differences seen between their mechanisms was dependent on the population group from which they arose (Decuypere *et al*., 2012). Interestingly there were parallels from this report and the resistant parasites selected for in this thesis in that no significant difference in thiols was noted between resistant and sensitive pathogens and stage-specific resistance was detected (Section 4.5)

Downregulation or loss of the putative reductases, TDR1 and LmACR2 has not been looked for in clinical isolates to the same extent as the mechanisms mentioned above. This is not surprising as almost all of the *in vitro* data on resistance published thus far has been generated using strains selected for resistance by trivalent metalloids. There are contradictory results on the possible reductases in clinical isolates from the Indian subcontinent with one group looking at Nepalese isolates seeing no change in expression of TDR1 or LmACR2 and another looking at isolates from Bihar seeing a 26

fold over expression of TDR1 in resistant vs sensitive isolates (Mukhopadhyay *et al*., 2011). If TDR1 is upregulated and it is the main antimonial reductase one would expect to see parasites that are hypersensitive to Sb^V not resistant. These contradictions may be due the polyclonal nature of antimonial resistance as described above and it would be interesting to know if either groups displayed stage-specific resistance. An absence of change in TDR1 regulation in Brazilian isolates between patients who succeeded or failed with antimonial treatment has also been observed (Torres *et al*., 2010). There may be other antimonial reductases not yet identified.

Thus, there are many ways in the Indian Subcontinent that antimonial resistance could be selected for: through sub therapeutic exposure to arsenic in exposed patients: through inadequate dose of antimonials; through pentavalent antimonial preparations contaminated with trivalent forms (although this should be improved since the IDA regulations (Section 1.2.2)); through inadequate length of regime; and through incomplete cure and selection for resistance in an optimally dosed patient of resistance within the patient. These different ways to reach resistance may arise from mutations or from a mixed population of parasites within a patient and may select for different resistance mechanisms.

It is possible that in Africa and South America the increase in antimonial dosing to 20 mg kg^{-1} and longer duration courses occurred faster than in India, partially in response to reports of resistance from India, as well as a response to local failure rates. This would have decreased the risk of selection of resistant parasites. Although there is an additional mechanisms for development of resistance in South America in the often non curative treatment of dogs with pentavalent antimonials (Gramiccia *et al*., 1992) this has not resulted in high treatment failure rates in humans and antimonials are still recommended as treatment in this region (World Health Organization, 2010). Another

potential reason for the difference in resistance rates are developmental differences between the species *L.donovani* and *L.infantum* but more work is needed to answer this hypothesis.

There has been a consistent observation that antimonial resistant isolates are stable and fitter than antimonial sensitive isolates (Vanaerschot *et al*., 2011; Vanaerschot *et al*., 2010). The presence of such parasites allows for the spread of resistance through the Indian subcontinent even if only some of the above resistance scenarios lead to stable fitter parasites. Mathematical models of resistance demonstrate that antimonial resistance alone cannot explain the dramatic spread of resistance in Bihar. The picture favours an increased transmissibility fitness rather than disease related fitness and the model, taking into account multiple emergences of resistance, predicts that antimonial resistant parasites will replace antimonial sensitive parasites within 20 to 40 years after the first occurrence of treatment failure (Stauch *et al*., 2012).

4.6 Antimonial treatment outcomes in the field

4.6.1 Antimonial treatment failure

The primary objective of my field work was to evaluate if arsenic exposure is related to treatment failure in SSG treated patients, thus supporting the finding of the *in vivo* study (Section 3.6) that SSG- resistant *Leishmania* parasites can be selected for by exposure to oral arsenic. The results from the field study, although they indicate a trend towards increased treatment failure in arsenic exposed patients, have failed to provide robust evidence in support of this mechanism.

A number of reasons may explain why the field results do not support the laboratory results. Firstly, the numbers of patients available were limited as SSG treatment for VL is no longer recommended in Bihar and, consequently, the study was

underpowered to detect a low level of risk. Secondly, the *in vivo* experiments were performed in the upper range of arsenic levels found in Bihar. Only 2 patients in our study had arsenic levels in their local water supplies in this range and, interestingly, they both had a successful treatment with SSG. Thirdly, treatment failure from SSG is thought to be multifactorial and not only due to resistant parasites, particularly in that there is low correlation between *in vitro* resistance tests and clinical outcome (Rijal *et al*., 2007). Additionally, extensive work on SSG-resistant parasites has shown that they have increased fitness and virulence when compared to SSG-sensitive strains indicating that they would thus be preferentially transmitted in the field (Vanaerschot *et al*., 2011). These latter two reasons could mask any existing relationship between arsenic exposure and SSG treatment failure in VL.

The two patients with high arsenic levels but good response to antimonial treatment warrant further discussion. One was a ten year old girl who died 2 years following treatment from 'asthma' which may also have been arsenic associated lung disease/bronchiectasis (Section 1.3.6.3). Liver disease with associated splenomegaly has also been described in arsenic exposed persons (Mazumder, 2005) which could lead to a misdiagnosis of kala azar as rK39 can be falsely positive in an endemic population – this would explain the 'success' of SSG if the fever wasn't initially caused by *Leishmania.* This patient only had a 24 day delay to treatment and another explanation is that, in view of the faster elimination of arsenic in children (Chowdhury *et al*., 2003), this may not have been long enough for the *Leishmania* parasite to build resistance. The other patient was a 37 year old male who died 3 months prior to the study in a road traffic accident and was diagnosed with TB subsequent to his VL episode so it is also possible here that there was a misdiagnosis.

Although multiple studies have been carried out on the mechanisms of antimonial resistance in the *Leishmania* parasite (Croft *et al*., 2006), only a few epidemiological studies have been performed looking at the clinical and demographic risk factors associated with SSG treatment failure in the leishmaniases (Llanos-Cuentas *et al*., 2008; Rijal *et al*., 2010). The most relevant is a prospective study in Nepal on VL patients (Rijal *et al*., 2010) that identified patients living on the border of Bihar as having a markedly increased chance of treatment failure. Additionally, fever over 12 weeks, interruption of treatment and ambulatory treatment were strong risk factors for treatment failure. All of the patients in our study received ambulatory treatment, treatment interruption was not specifically assessed and no association was found between prolonged fever and treatment failure. As arsenic contamination is an issue also in Nepal, it would be interesting to assess retrospectively the arsenic exposure in this cohort of patients.

A study in Peru of SSG treatment failure (Llanos-Cuentas *et al*., 2008) in CL identified age and recent relocation into an endemic area as relevant risk factors. *Leishmania* species, number of lesions and lesion diameter are additional risk factors for adverse outcome that are specific to CL alone. Arsenic contamination from mining is a significant issue in Peru (Reuer *et al*., 2012) and as arsenic accumulates in the skin as well as liver and spleen the hypothesis of arsenic exposure generating resistance is relevant to CL as well as VL. Interestingly the area, La Oroya where arsenic contamination from mining is an issue appears to be in a similar geographic location to a group of antimonial resistant CL isolates tested in 2006 (Yardley *et al*., 2006). Further work is underway in this area of Peru to establish if there is a link between arsenic exposure and antimonial treatment failure.

A study on rural VL care in Muzaffarpur district, Bihar (Hasker *et al*., 2010), where no significant arsenic contamination has been detected (Perry *et al*., 2011) but SSG resistance is well established (Sundar *et al*., 2000), had a SSG failure rate of 40% compared with 59% in our study. The difference in failure rates in these similar communities, with comparable district level literacy rates of 61.9% and 63.45% respectively (Census Organization of India, 2011) may be, among other factors, attributable to arsenic exposure and the increased mortality rate.

4.6.2 Mortality in the antimonial treated cohort

The main finding in this study, the strong relationship found between arsenic exposure and all-cause and VL mortality, agrees with a population-based cohort study performed in Bangladesh (Sohel *et al*., 2009) that demonstrated an increased risk for both all-cause mortality and infectious disease deaths with increasing arsenic exposure. The risk of death from VL in arsenic-exposed persons in our cohort is predominantly present greater than 3 months after the commencement of symptoms. This could be explained by 3 disease mechanisms: 1) the longer incubation time in arsenic-exposed tissues would allow the parasites to develop arsenic tolerance and thus SSG resistance leading to ineffective treatment; 2) patients being infected with SSG-resistant parasites (Sundar, 2001); and 3) the risk of mortality increases with the progressive dampening effect of VL on the immune system (Kaye and Aebischer, 2011) combined with the immunotoxicity of arsenic exposure (Dangleben *et al*., 2013). An alternative explanation is with arsenic exposure combined with antimonial treatment increases the risk of toxicity from antimonial compounds. The opinion of the experienced VL clinician Shyam Sundar is that deaths that occur early in a treatment course are usually related to disease and not to antimonial toxicity (personal communication Shyam Sundar). The timing of death from toxicity may be different in arsenic contaminated

patients but there is no experimental evidence for this. The characteristics of the 16 patients who died from VL are shown in Table 3.10 and demonstrate that the deaths occurred at varying points in the treatment course and there is no particular difference in timing between the exposed and unexposed groups.

The rural VL study in Muzaffarpur referred to above (Hasker *et al*., 2010) only reported one death (0.7%) of undefined cause in a cohort of 138 (68 of whom were treated with SSG) compared with 21 deaths (19%) in our 110 cohort with 16 directly as a result of VL (14.5%). This could be explained by a number of factors as well as arsenic exposure such as: a different quality of primary health care between districts; a more virulent parasite population; or a general lower immune status, for example due to malnutrition, in our Samastipur study cohort. However, if the immunotoxicity of arsenic and VL combined is responsible for the increased death rate in our study then arsenic exposure may increase mortality from VL with treatments other than SSG. This warrants further research.

Previous studies on mortality in VL have identified the extremes of age, (<5 and > 45 years old), long duration of symptoms, co-infections and laboratory abnormalities such as severe anaemia and jaundice as risk factors for death (Madalosso *et al*., 2012; Mueller *et al*., 2009; Sampaio *et al*., 2010; van den Bogaart *et al*., 2013). Due to the retrospective design of our study, and the rural management of the VL cases, information on the above clinical risk factors is mainly not available. Our data does agree with age-associated risk at the extremes of age but no increased mortality risk was seen with delay to first SSG treatment.

Could arsenic be silently changing the epidemiology of VL in exposed regions? This work shows it may have an effect on mortality. It would require a very large study to answer the question of whether arsenic contamination affects the incidence of VL and

the proportion of asymptomatic to clinical cases. This would be particularly difficult to answer as VL epidemiology is migratory (Malaviya *et al*., 2011) and the reasons for progression to clinical infection are poorly understood (Singh *et al*., 2014). From an immunological perspective cell mediated immunity is thought to be integral to developing clinical infection (Bern *et al*., 2010), a defence which is significantly reduced in arsenic-exposed persons (Ahmed *et al*., 2014).

4.6.3 Limitations of field work study

The retrospective design of this study had some limitations in ascertaining VL cases and treatments used. There was generally poor documentation of diagnostic methods, treatment duration and dosing. No parasitological confirmation was available, however, most of the patients were diagnosed with VL by the rK39 rapid diagnostic test which shows a good specificity (90.6%) in the context of clinically suspected disease (Chappuis *et al*., 2006). Relying on patient's and relatives' responses increased the risk of recall bias (Hassan, 2005). Therefore, the definition of treatment failure included the requirement for another treatment course which is more concrete that a subjective analysis of symptoms. As SSG treatment is prolonged and painful with a dramatic economic impact on the patient's family, the recall bias for treatment may be limited. However, it is possible that administered doses were too low, or treatments were given with unrecalled interruptions that could have impacted on treatment outcome. Unfortunately, there was generally poor documentation of diagnostic methods, treatment duration and dosing.

Finally, VL as a cause of death was confirmed by verbal autopsy only carried out by one physician. For this reason VL mortality is always presented with all-cause mortality. Verbal autopsy has been criticised as a method of ascertaining cause of death (Butler, 2010; Soleman *et al*., 2006) due to broad generalisation and over-diagnosis of

infectious conditions with non-specific symptoms eg. malaria. However in this setting, these issues are less prevalent, as many of our patients were on a register as having VL and for all patients we had at least verbal communication that a physician had diagnosed VL.

A substantial design difficulty in this study is the timing of the assessment of arsenic exposure. Forty percent of the patients had their tube well inserted in the years following their treatment episode and therefore a proxy of their arsenic exposure had to be created from the average of 5 wells surrounding their living area. Additionally, the level of arsenic contamination in a tube well water sample can vary according to prior usage that day, depth of well and age of well (Erickson and Barnes, 2006; Ravenscroft *et al*., 2009) adding further factors into the difficulty of building up an accurate picture of arsenic exposure at a historic time point. Although water arsenic levels were being collected on average 5 (± 1.3) years post treatment, it is generally assumed that arsenic in tube wells in the Bengal basin is either stable over time (BGS and DPHE, 2011; Cheng *et al*., 2005; Rahman *et al*., 2007; Sohel *et al*., 2009) or may rise gradually (Erickson and Barnes, 2006; Ravenscroft *et al*., 2009).

Measurement of arsenic levels in urine is usually an excellent biomarker of arsenic exposure (Concha *et al*., 2006) and allows for a direct individual assessment which includes any exposure from foods and other sources. However in the context of this study it has many disadvantages. Urine represents arsenic exposure in the preceding 24 h (Vahter, 1994) whereas we are interested in representing arsenic exposure at the time of the patient's VL episode up to 6 years prior to the study. This, coupled with the need to impute 41% (n=45) of these values to be able to analyse the full cohort, due to the unavailability of these VL patients, decreases the value of urine as an exposure variable.

Additionally there is controversy on how to present urine measurements. The ideal way to measure arsenic in urine is through a 24 h collection but this is often impractical in field study settings. Some studies use μg g^{-1} of creatinine to address this, however arsenic exposure has been shown to increase creatinine clearance which would distort results (Gamble and Liu, 2005). There is no definitive cut off for elevated arsenic in urine. In 2001, D.N.G. Mazumder recommended a level of > 50 μg L^{-1} urinary arsenic (Mazumder, 2001), but this was to diagnose 'arsenicosis' and is coupled with drinking water at >50 μg L^{-1}. However, drinking water contaminated with arsenic at levels between 10-50 μg L^{-1} in my study and in the literature is associated with pathology (Sohel *et al*., 2009). In view of this, a cut off of urinary arsenic levels above the normal range of 5- 40 μg l^{-1} (Mandal and Suzuki, 2002; Ravenscroft *et al*., 2009) was utilized in this study.

It would have been ideal to perform a prospective study where the parasites' sensitivity to pentavalent antimony *in vitro* in macrophages was correlated with current arsenic exposure and the clinical outcome of SSG treatment. However, due to the high rate of treatment failure with antimony and the recommendation of discontinuation of use, this type of prospective study was not possible. The retrospective nature of the study meant that parasite isolates from antimony-treated cases were not available. The presence of SSG-resistant parasites was reported earlier in the Samastipur District (Thakur *et al*., 2004). Determining if antimonial resistant parasites are still circulating in the study area today may have helped to interpret the findings of our study, provided that stably resistant parasites had not extensively displaced SSG-sensitive isolates due to their increased fitness (Vanaerschot *et al*., 2010).

4.6.4 Rural management of VL

This cohort study was performed in an area where research on VL has not been undertaken previously and gives a view of practice in an area without any special intervention. It highlights the issue, previously identified in Muzaffarpur (Hasker *et al*., 2010), that SSG was still being used in just under a quarter of patients treated between 2006 and 2010, with no record of its generally poor outcome, despite recommendations for its discontinuation since 2005 (Chappuis *et al*., 2007). In the registers available at the PHC there was no indication of any treatment failure or relapse and on interview the staff were only aware of one death during the study period. The majority of patients (57%) felt the need to change health care provider to obtain an effective treatment. The doctors at the PHC knew of the issue of SSG resistance and have been aware of a drive to mainly use miltefosine or amphotericin but, worryingly, were unaware of the VL elimination programme. They were aware of the issue of arsenic contamination but unaware of its wide-ranging health effects.

Two further issues identified during the field work were firstly that there is a substantial delay between the patient initially seeking care with a village health worker or unqualified ‘doctor’ and finally receiving treatment. In our study the median delay was 30 d. Prompt treatment reduces morbidity and mortality and work is ongoing to empower the village health workers to be able to identify kala azar and refer patients promptly and appropriately to the right health care facility.

Secondly, in India is that it is very easy to obtain a private tube well even for those below the poverty line and the Public Health and Education department have mainly focussed their surveys on governmental wells. Shallower wells are cheaper to obtain but unfortunately run a higher risk of arsenic contamination and their insertion is undocumented. In India, water supply is a state level responsibility but the provision of

clean water is not being matched by an adequate surveillance system. This work shows the urgent need for improved record keeping, education and intervention in both VL and arsenic contamination within these vulnerable communities (Boelaert *et al*., 2009; Sohel *et al*., 2009).

4.7 Arsenic hypothesis and summary

This study on arsenic, antimony and visceral leishmaniasis has looked in depth at the relationship between these 2 metalloids and this potentially fatal parasitic disease particularly in relation to the four steps of the hypothesis depicted in Figure 1.22.

In **step 1,** humans drink arsenic-contaminated water. Through our murine arsenic exposure model we have demonstrated that, as well as the established accumulation in skin, hair, nails and liver, arsenic also is detected at significant levels in the other organs related to *Leishmania*, namely the spleen and the bone marrow. This accumulation is related to dose and duration. At a cellular level we demonstrated that arsenic accumulates in macrophages, the cell in which the leishmania parasite resides.

We demonstrated in the field in Bihar that arsenic contamination is present in areas of VL endemicity. Twenty four percent of the VL patients in our cohort had a mean local arsenic contamination level of greater than the WHO limit of 10 µg L^{-1} and 4.5% a level greater than the Indian Bureau of National Standards limit[22] of 50 µg L^{-1}. However previous arsenic surveys have shown that levels vary considerably between regions[23].

It is important to note that humans are exposed to arsenic from many sources other than drinking water: food grown in arsenic contaminated soil can accumulate arsenic, arsenic in cosmetics can be absorbed through the skin, chicken feed can contain

[22] http://bis.org.in/sf/fad/FAD25(2047)C.pdf
[23] http://www.soesju.org/arsenic/arsenicContents.htm?f=groundwater_bihar.htm

high levels of organoarsenicals (although this is less relevant in the vegetarian Hindu communities of Bihar) (Mukhopadhyay *et al*., 2002) and arsenic has even been found in counterfeit drugs including miltefosine (personal communication Thomas Dorlo).

In **step 2,** the leishmania parasite is selected for resistance to arsenic in the human body.

We demonstrated the selection of cross resistance to antimony through exposure to arsenic *in vitro* and *in vivo.* The *in vivo* oral exposure led to levels of arsenic in liver, spleen and bone marrow that were capable of inducing resistance in the leishmania parasite. This *in vivo* generated resistance was demonstrated using *ex vivo* amastigotes in an *in vitro* in macrophage assay but was found to be stage-specific as the corresponding promastigotes were sensitive to trivalent metalloids.

It is unlikely that the leishmania parasite would have been exposed to arsenic within the sand fly stages of the life cycle. Sand flies eat aphid honey dew as their sugar source and the female takes blood meals. Aphid honey dew is unlikely to contain arsenic. If the sand fly is taking blood meals from arsenic exposed patients then there will be arsenic in the gut. In mammals arsenic is rapidly absorbed from the gut and should the same apply in sand flies, then exposure of a growing promastigote population in the gut would be minimal. A sand fly larva's main food source is thought to be rat faeces (Section 1.1.8.1) and even if rats are drinking arsenic contaminated water it would be excreted in the urine and not the faeces.

In **step 3,** treatment failure with antimony occurs secondary to selection of resistance through exposure to arsenic. This was demonstrated in our *in vivo* model through a minimal suppression of parasite growth (<20%) following a 5 day Sb^V treatment course in the arsenic exposed groups compared with a > 75% suppression in the non-exposed group. Hepatic levels of arsenic and antimony measured post treatment

indicated that arsenic interfering with the metabolism of antimony was unlikely to have contributed to the treatment failure in the *in vivo* model. The immunosuppressive effect of arsenic however may have decreased the effect of the immunostimulatory effect of antimony but we have no experimental data to support this.

In the field there was a trend of increased risk of treatment failure in arsenic-exposed patients. However this trend was not significant. It is not clear whether the levels of arsenic exposure in this community were not high enough to induce resistance or the shortcomings in study timing and design meant that we were unable to detect a link that may be present (Section 4.6.3).

In **step 4,** the selected resistant parasite population is propagated through the leishmania vulnerable community due to stability of resistance and increased fitness over sensitive parasites. We demonstrated stability of resistance in the amastigotes of our arsenic *in vivo* generated resistant line over >8 months. Apart from a slightly higher parasite load at the end of the 5th passage in the 10 ppm exposed group there was no experimental evidence of increased fitness in our parasite lines. However, this has been demonstrated in clinical isolates (Vanaerschot *et al*., 2011; Vanaerschot *et al*., 2010).

4.8 Further work

The work presented here in this thesis provides many questions for future research:

Firstly, the presence of a leishmania antimony reductase that is lost in antimonial resistant parasites has not yet been established. Using the resistant leishmania parasites selected for by the in-macrophage exposure assay and the *in vivo* arsenic exposure model and a combination of genomic sequencing and western blot analysis with newly generated antibodies this may be possible. However even if a reductase was identified that was also common to all antimonial resistant clinical isolates it still may not be able

to provide a universal test for antimonial treatment failure as resistant parasites can be isolated from patients who experience clinical cure.

Secondly, is there an association with antimonial treatment failure and arsenic in cutaneous leishmaniasis patients? Arsenic is known to accumulate to the skin and therefore leishmania parasites in CL lesions could be exposed to high levels of arsenic. In Peru a prospective study of CL looking at reasons for antimonial treatment failure is now gathering information on arsenic exposure of recruited patients.

Thirdly, does the increased risk of death from VL with arsenic exposure hold for patients not treated with antimonials? The increased risk of death from VL in arsenic exposed patients may be secondary to arsenic's immunosuppressive effects. It may therefore hold that arsenic exposure increases the risk of death in patients treated with any anti-leishmanial agent.

Fourthly, how does arsenic exposure affect outcome in other infectious diseases? The immunosuppressive actions and toxic effect of arsenic on the macrophage may mean that exposure has an adverse effect on treatment outcome and mortality for other pathogens, particularly those which are obligate intracellular pathogens. Analysis of large scale data sets of mortality from Bangladesh is underway to begin to address this question.

4.9 Conclusions

Nepal and Bihar are the only places worldwide where arsenic contamination has been detected, VL is endemic and there are such high levels of resistance that antimonial compounds are no longer recommended for use. This work demonstrates that it is biochemically and epidemiologically plausible that treatment failure could be linked to arsenic exposure. However, it also shows that antimonial resistance is easily selected

for by Sb^V in macrophage. The Indian health care system is under-regulated in both the public and private sectors and it is likely that the number of sub-therapeutic or incomplete courses are higher in this continent than in Africa where the majority of VL treatment is administered by aid organisations such as MSF and in Brazil where VL care is free and given at hospital level (Alvar *et al*., 2012). Additionally the cost of treatment in India is the highest per gross national income which is a significant barrier to completion of a full treatment course (Thornton *et al*., 2010). Thus, in conclusion, although there are many routes to antimonial resistance and treatment failure in Bihar, it is possible that exposure of the population to arsenic through contamination of the groundwater has been contributory.

A recent global review of the epidemiology of the leishmaniases gave an overall case fatality rate for VL of 10 % (Alvar *et al*., 2012) which is considerably lower than the VL mortality rate of 14.5 % in the field work presented here. The field study demonstrates that arsenic exposure is strongly associated with VL mortality in this antimony treated cohort and may be responsible for the elevated mortality rate. Arsenic contamination is a significant issue in Bihar and effort needs to be made to improve coordination of the mitigation programmes with a push for the Bureau of Indian Standards to bring down its acceptable limit to < 10 µg L^{-1} in accordance with the WHO guidelines.

This research into antimonial resistance and treatment failure has important implications for the leishmaniases worldwide and is a reminder to consider the environment in which an organism is propagating when assessing reasons for treatment failure and mortality.

4.10 Lessons learned

The work presented in this thesis attempted to answer the question of how arsenic may have contributed to the emergence of antimonial treatment failure in Bihar using many different methods. Looking back on 3-4 years of work there will always be areas that you wish you could change or plans that would have been different if you had been able to make them with the knowledge and experience that you have at the end of that time period.

Presenting this work together highlights the issues of translating between the basic science laboratory and the field, particularly those of dose equivalence between mice and men. It would have been more helpful in answering the hypothesis underlying this work to have included a lower dose of arsenic exposure in the mouse model that is more representative of levels commonly found in Bihar rather than the lowest level of murine exposure only being at the very upper range of arsenic levels in the field. Additionally, in this *in vivo* work, selecting for resistance in triplicate at each arsenic exposure level would have given us parasite isolates that would be able to sequenced, allowing for more detailed analysis for resistance mechanisms.

The field study performed had many limitations which are addressed at length in the discussion and demonstrated the issues of trying to answer a question about an event that has happened maybe 20 years ago or more. This type of field study would not be able to distinguish between resistance driven by arsenic exposure and resistance by antimony therapy but instead aimed to look for whether arsenic exposure and antimonial treatment failure were linked. With the small numbers in the cohort it would only have been able to detect a very strong association between arsenic exposure and treatment failure with the hypothesis that the presence of arsenic has a high probability

to select for mutations that lead to antimonial resistance, as demonstrated in the murine model.

Epidemiological studies generally only show associations and it is a difficult task to ever 'prove' causality. As it was, although no significant association between arsenic exposure and treatment failure was found, our study detected an unexpectedly strong relationship between increased mortality from VL in those exposed to arsenic. A more optimum design could have been to have had 2 study sites – one with arsenic exposure and the other without and compare treatment failure and mortality. Additional data collected on nutritional status and socioeconomic status of patients would have strengthened the analysis of confounding factors.

As discussed above, I hope that the field study will act as a pilot for further investigation into the relationship between arsenic exposure, treatment failure and mortality in both VL and other infectious pathogens.

Reference List

Abok, K., Fredriksson, B.A. and Brunk, U. (1988). An experimental model system for leishmaniasis. An ultrastructural study on cultured macrophages exposed to Leishmania parasites and sodium stibogluconate. *APMIS 96*, 589-595.

Adaui, V., Schnorbusch, K., Zimic, M., Gutierrez, A., Decuypere, S., Vanaerschot, M., De, D.S., Maes, I., Llanos-Cuentas, A., Chappuis, F., Arevalo, J. and Dujardin, J.C. (2011). Comparison of gene expression patterns among *Leishmania braziliensis* clinical isolates showing a different in vitro susceptibility to pentavalent antimony. *Parasitology 138*, 183-193.

Ahamed, S., Kumar, S.M., Mukherjee, A., Amir, H.M., Das, B., Nayak, B., Pal, A., Chandra, M.S., Pati, S., Nath, D.R., Chatterjee, G., Mukherjee, A., Srivastava, R. and Chakraborti, D. (2006). Arsenic groundwater contamination and its health effects in the state of Uttar Pradesh (UP) in upper and middle Ganga plain, India: a severe danger. *Sci. Total Environ. 370*, 310-322.

Ahlborn, G.J., Nelson, G.M., Grindstaff, R.D., Waalkes, M.P., Diwan, B.A., Allen, J.W., Kitchin, K.T., Preston, R.J., Hernandez-Zavala, A., Adair, B., Thomas, D.J. and Delker, D.A. (2009). Impact of life stage and duration of exposure on arsenic-induced proliferative lesions and neoplasia in C3H mice. *Toxicology 262*, 106-113.

Ahmed, M.F., Ahuja, S., Alauddin, M., Hug, S.J., Lloyd, J.R., Pfaff, A., Pichler, T., Saltikov, C., Stute, M. and van, G.A. (2006). Epidemiology. Ensuring safe drinking water in Bangladesh. *Science 314*, 1687-1688.

Ahmed, S., Moore, S.E., Kippler, M., Gardner, R., Hawlader, M., Wagatsuma, Y., Raqib, R. and Vahter, M. (2014). Arsenic exposure and cell-mediated immunity in pre-school children in rural Bangladesh. *Toxicol. Sci.*

Ait-Oudhia, K., Gazanion, E., Vergnes, B., Oury, B. and Sereno, D. (2011). Leishmania antimony resistance: what we know what we can learn from the field. *Parasitology Research 109*, 1225-1232.

Al Olayan, E.M., Beetsma, A.L., Butcher, G.A., Sinden, R.E. and Hurd, H. (2002). Complete development of mosquito phases of the malaria parasite in vitro. *Science 295*, 677-679.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1983). Molecular biology of the cell. (New York: Garland Publishing).

Albrich, W.C., Monnet, D.L. and Harbarth, S. (2004). Antibiotic selection pressure and resistance in *Streptococcus pneumoniae* and *Streptococcus pyogenes*. *Emerging Infectious Diseases 10*, 514-517.

Alcorn, T. (2012). Antibiotic use in livestock production in the USA. *Lancet Infectious Diseases 12*, 273-274.

Alderton, W.K., Cooper, C.E. and Knowles, R.G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal 357*, 593-615.

Ali, K.S., Rees, R.C., Terrell-Nield, C. and Ali, S.A. (2013). Virulence loss and amastigote transformation failure determine host cell responses to *Leishmania mexicana*. *Parasite Immunol. 35*, 441-456.

Allen, S. and Neal, R. A. (1989). The *in vitro* suceptibility of macrophages infected with amastigotes of *Leishmania spp.* to pentavalent antimonial drugs and other compounds with specila reference to cutaneous isolates. In *Leishmaniasis. The Current Status and New Strategies for Control*, D.T. Hart, ed. (New York: Plenum Press), pp. 711-720.

Alvar, J., Croft, S.L., Kaye, P., Khamesipour, A., Sundar, S. and Reed, S.G. (2013). Case study for a vaccine against leishmaniasis. *Vaccine 31*, B244-B249.

Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J. and den, B.M. (2012). Leishmaniasis worldwide and global estimates of its incidence. *PLoS. One. 7*, e35671.

Andrew, A.S., Jewell, D.A., Mason, R.A., Whitfield, M.L., Moore, J.H. and Karagas, M.R. (2008). Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a U.S. population. *Environmental Health Perspectives 116*, 524-531.

Argos, M., Kalra, T., Rathouz, P.J., Chen, Y., Pierce, B., Parvez, F., Islam, T., Ahmed, A., Rakibuz-Zaman, M., Hasan, R., Sarwar, G., Slavkovich, V., van Geen, A., Graziano, J. and Ahsan, H. (2010). Arsenic exposure from drinking water, and all-cause and chronic-disease mortalities in Bangladesh (HEALS): a prospective cohort study. *Lancet 376*, 252-258.

Argos, M., Kibriya, M.G., Parvez, F., Jasmine, F., Rakibuz-Zaman, M. and Ahsan, H. (2006). Gene expression profiles in peripheral lymphocytes by arsenic exposure and skin lesion status in a Bangladeshi population. *Cancer Epidemiol. Biomarkers Prev. 15*, 1367-1375.

Arnesen, T. and Nord, E. (1999). The value of DALY life: problems with ethics and validity of disability adjusted life years. *British Medical Journal 319*, 1423-1425.

Bachmanov, A.A., Reed, D.R., Beauchamp, G.K. and Tordoff, M.G. (2002). Food intake, water intake, and drinking spout side preference of 28 mouse strains. *Behavior Genetics 32*, 435-443.

Balasegaram, M., Ritmeijer, K., Lima, M.A., Burza, S., Ortiz, G.G., Milani, B., Gaspani, S., Potet, J. and Chappuis, F. (2012). Liposomal amphotericin B as a treatment for human leishmaniasis. *Expert Opinion on Emerging Drugs 17*, 493-510.

Banerjee, N., Banerjee, S., Sen, R., Bandyopadhyay, A., Sarma, N., Majumder, P., Das, J.K., Chatterjee, M., Kabir, S.N. and Giri, A.K. (2009). Chronic Arsenic Exposure Impairs Macrophage Functions in the Exposed Individuals. *Journal of Clinical Immunology 29*, 582-594.

Barrett, M.P. (2010). Potential new drugs for human African trypanosomiasis: some progress at last. *Current Opinion in Infectious Diseases 23*, 603-608.

Basu, A., Mitra, S., Chung, J., Guha Mazumder, D.N., Ghosh, N., Kalman, D., von Ehrenstein, O.S., Steinmaus, C., Liaw, J. and Smith, A.H. (2011). Creatinine, diet,

micronutrients, and arsenic methylation in West Bengal, India. *Environmental Health Perspectives 119*, 1308-1313.

Bates, P.A. (2007). Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. *International Journal for Parasitology 37*, 1097-1106.

Berg, M., Stengel, C., Pham, T.K., Pham, H.V., Sampson, M.L., Leng, M., Samreth, S. and Fredericks, D. (2007). Magnitude of arsenic pollution in the Mekong and Red River Deltas--Cambodia and Vietnam. *Sci. Total Environ. 372*, 413-425.

Berman, J.D., Gallalee, J.F. and Gallalee, J.V. (1988). Pharmacokinetics of pentavalent antimony (Pentostam) in hamsters. *American Journal of Tropical Medicine and Hygiene 39*, 41-45.

Berman, J.D., Gallalee, J.V. and Hansen, B.D. (1987). *Leishmania mexicana* uptake of sodium stibogluconate (pentostam) and pentamidine by parasite and macrophages. *Experimental Parasitology 64*, 127-131.

Bern, C., Courtenay, O. and Alvar, J. (2010). Of cattle, sand flies and men: a systematic review of risk factor analyses for South Asian visceral leishmaniasis and implications for elimination. *PLoS Neglected Tropical Diseases 4*, e599.

Bern, C., Haque, R., Chowdhury, R., Ali, M., Kurkjian, K.M., Vaz, L., Amann, J., Wahed, M.A., Wagatsuma, Y., Breiman, R.F., Williamson, J., Secor, W.E. and Maguire, J.H. (2007). The epidemiology of visceral leishmaniasis and asymptomatic leishmanial infection in a highly endemic Bangladeshi village. *American Journal of Tropical Medicine and Hygiene 76*, 909-914.

BGS and DPHE (2011). Changes with time: groundwater monitoring. In *Arsenic contamination of groundwater in Bangladesh*, D.G. Kinniburgh and P.L. Smedley, eds. (Keyworth: British Geological Survey), pp. 175-185.

Bhattacharjee, H., Carbrey, J., Rosen, B.P. and Mukhopadhyay, R. (2004). Drug uptake and pharmacological modulation of drug sensitivity in leukemia by AQP9. *Biochemical and Biophysical Research Communications 322*, 836-841.

Bishayi, B. and Sengupta, M. (2003). Intracellular survival of *Staphylococcus aureus* due to alteration of cellular activity in arsenic and lead intoxicated mature Swiss albino mice. *Toxicology 184*, 31-39.

Biswas, R., Ghosh, P., Banerjee, N., Das, J.K., Sau, T., Banerjee, A., Roy, S., Ganguly, S., Chatterjee, M., Mukherjee, A. and Giri, A.K. (2008). Analysis of T-cell proliferation and cytokine secretion in the individuals exposed to arsenic. *Human & Experimental Toxicology 27*, 381-386.

Boelaert, M., Meheus, F., Sanchez, A., Singh, S.P., Vanlerberghe, V., Picado, A., Meessen, B. and Sundar, S. (2009). The poorest of the poor: a poverty appraisal of households affected by visceral leishmaniasis in Bihar, India. *Tropical Medicine and International Health 14*, 639-644.

Brahmachari, U.N. (1989). Chemotherapy of antimonial compounds in kala-azar infection. Part IV. Further observations on the therapeutic values of urea stibamine. By U.N. Brahmachari, 1922. *Indian Journal of Medical Research 89*, 393-404.

Brochu, C., Haimeur, A. and Ouellette, M. (2004). The heat shock protein HSP70 and heat shock cognate protein HSC70 contribute to antimony tolerance in the protozoan parasite *Leishmania*. *Cell Stress & Chaperones 9*, 294-303.

Brochu, C., Wang, J.Y., Roy, G., Messier, N., Wang, X.Y., Saravia, N.G. and Ouellette, M. (2003). Antimony uptake systems in the protozoan parasite *Leishmania* and accumulation differences in antimony-resistant parasites. *Antimicrobial Agents and Chemotherapy 47*, 3073-3079.

Brotherton, M.C., Bourassa, S., Leprohon, P., Legare, D., Poirier, G.G., Droit, A. and Ouellette, M. (2013). Proteomic and genomic analyses of antimony resistant *Leishmania infantum* mutant. *Plos One 8*, e81899.

Brouns, M, Janssen, M, and Wong, A L Z (2013). *Dealing with arsenic in rural Bihar, India. Evaluating the successes and failures of mitigation projects and providing a long-term mitigation strategy***.** PhD Thesis, Deft University of Technology, 122 pp.

Brunsting, J.H. and McBean, E.A. (2014). In situ treatment of arsenic-contaminated groundwater by air sparging. *J. Contam Hydrol. 159*, 20-35.

Bucheton, B., Kheir, M.M., El-Safi, S.H., Hammad, A., Mergani, A., Mary, C., Abel, L. and Dessein, A. (2002). The interplay between environmental and host factors during an outbreak of visceral leishmaniasis in eastern Sudan. *Microbes and Infection 4*, 1449-1457.

Burza, S., Sinha, P.K., Mahajan, R., Lima, M.A., Mitra, G., Verma, N., Balasegarem, M. and Das, P. (2014a). Five-year field results and long-term effectiveness of 20 mg/kg liposomal amphotericin B (Ambisome) for visceral leishmaniasis in Bihar, India. *PLoS Neglected Tropical Diseases 8*, e2603.

Burza, S., Sinha, P.K., Mahajan, R., Lima, M.A., Mitra, G., Verma, N., Balsegaram, M. and Das, P. (2014b). Risk factors for visceral leishmaniasis relapse in immunocompetent patients following treatment with 20 mg/kg liposomal amphotericin B (Ambisome) in Bihar, India. *PLoS Neglected Tropical Diseases 8*, e2536.

Butler, D. (2010). Verbal autopsy methods questioned. *Nature 467*, 1015.

Callahan, H.L., Portal, A.C., Devereaux, R. and Grogl, M. (1997). An axenic amastigote system for drug screening. *Antimicrobial Agents and Chemotherapy 41*, 818-822.

Carew, M.W., Naranmandura, H., Shukalek, C.B., Le, X.C. and Leslie, E.M. (2011). Monomethylarsenic diglutathione transport by the human multidrug resistance protein 1 (MRP1/ABCC1). *Drug Metab Dispos. 39*, 2298-2304.

Carter, K.C., Hutchison, S., Boitelle, A., Murray, H.W., Sundar, S. and Mullen, A.B. (2005). Sodium stibogluconate resistance in *Leishmania donovani* correlates with greater tolerance to macrophage antileishmanial responses and trivalent antimony therapy. *Parasitology 131*, 747-757.

Census Organization of India. (2011). Indian Census 2011.

Cervantes, C., Ji, G.Y., Ramirez, J.L. and Silver, S. (1994). Resistance to arsenic compounds in microorganisms. *FEMS Microbiology Reviews 15*, 355-367.

Chakraborti, D., Mukherjee, S.C., Pati, S., Sengupta, M.K., Rahman, M.M., Chowdhury, U.K., Lodh, D., Chanda, C.R., Chakraborty, A.K. and Basul, G.K. (2003). Arsenic groundwater contamination in Middle Ganga Plain, Bihar, India: A future danger? *Environmental Health Perspectives 111*, 1194-1201.

Chang, C.S. and Chang, K.-P. (1985). Heme requirement and acquisition by extracellular and intracellular stages of *Leishmania mexicana amazonensis*. *Mol. Biochem. Parasitol. 16*, 267-276.

Chang, K.P. (1979). *Leishmania donovani*: promastigote-macrophage surface interactions in vitro. *Experimental Parasitology 48*, 175-189.

Chang, K.P. and Dwyer, D.M. (1978). *Leishmania donovani*. Hamster macrophage interactions in vitro: cell entry, intracellular survival, and multiplication of amastigotes. *Journal of Experimental Medicine 147*, 515-530.

Chappuis, F., Rijal, S., Soto, A., Menten, J. and Boelaert, M. (2006). A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *British Medical Journal 333*, 723.

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R.W., Alvar, J. and Boelaert, M. (2007). Visceral leishmaniasis: What are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology 5*, 873-882.

Chen, C.J., Chuang, Y.C., Lin, T.M. and Wu, H.Y. (1985). Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: high-arsenic artesian well water and cancers. *Cancer Research 45*, 5895-5899.

Chen, C.J., Kuo, T.L. and Wu, M.M. (1988). Arsenic and cancers. *Lancet 1*, 414-415.

Chen, Y.C., Guo, Y.L., Su, H.J., Hsueh, Y.M., Smith, T.J., Ryan, L.M., Lee, M.S., Chao, S.C., Lee, J.Y. and Christiani, D.C. (2003). Arsenic methylation and skin cancer risk in southwestern Taiwan. *J. Occup. Environ. Med. 45*, 241-248.

Cheng, Z., van, G.A., Seddique, A.A. and Ahmed, K.M. (2005). Limited temporal variability of arsenic concentrations in 20 wells monitored for 3 years in Araihazar, Bangladesh. *Environmental Science & Technology 39*, 4759-4766.

Chowdhury, U.K., Rahman, M.M., Sengupta, M.K., Lodh, D., Chanda, C.R., Roy, S., Quamruzzaman, Q., Tokunaga, H., Ando, M. and Chakraborti, D. (2003). Pattern of excretion of arsenic compounds [arsenite, arsenate, MMA(V), DMA(V)] in urine of children compared to adults from an arsenic exposed area in Bangladesh. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng 38*, 87-113.

Chulay, J.D., Fleckenstein, L. and Smith, D.H. (1988). Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. *Transactions of the Royal Society of Tropical Medicine and Hygiene 82*, 69-72.

Concha, G., Nermell, B. and Vahter, M. (2006). Spatial and temporal variations in arsenic exposure via drinking-water in Northern Argentina. *Journal of Health Population and Nutrition 24*, 317-326.

Cota, G.F., de Sousa, M.R., Fereguetti, T.O. and Rabello, A. (2013). Efficacy of anti-leishmania therapy in visceral leishmaniasis among HIV infected patients: a systematic review with indirect comparison. *PLoS Neglected Tropical Diseases 7*, e2195.

Croft, S.L. (2008). Kinetoplastida: new therapeutic strategies. *Parasite-Journal de la Societe Francaise de Parasitologie 15*, 522-527.

Croft, S.L., Neame, K.D. and Homewood, C.A. (1981). Accumulation of (^{125}Sb)sodium stibogluconate by *Leishmania mexicana amazonensis* and *Leishmania donovani in vitro*. *Comparative Biochemistry and Physiology - C Comparative Pharmacology and Toxicology 68*, 95-98.

Croft, S.L., Sundar, S. and Fairlamb, A.H. (2006). Drug Resistance in Leishmaniasis. *Clinical Microbiology Reviews 19*, 111-126.

da Luz, R.I., Vermeersch, M., Dujardin, J.C., Cos, P. and Maes, L. (2009). *In vitro* sensitivity testing of *Leishmania* clinical field isolates: preconditioning of promastigotes enhances infectivity for macrophage host cells. *Antimicrobial Agents and Chemotherapy 53*, 5197-5203.

Dangleben, N.L., Skibola, C.F. and Smith, M.T. (2013). Arsenic immunotoxicity: a review. *Environmental Health 12*, 73.

Das, S., Santra, A., Lahiri, S. and Mazumder, D.N.G. (2005). Implications of oxidative stress and hepatic cytokine (TNF-alpha and IL-6) response in the pathogenesis of hepatic collagenesis in chronic arsenic toxicity. *Toxicology and Applied Pharmacology 204*, 18-26.

De Rycker, M., Hallyburton, I., Thomas, J., Campbell, L., Wyllie, S., Joshi, D., Cameron, S., Gilbert, I.H., Wyatt, P.G., Frearson, J.A., Fairlamb, A.H. and Gray, D.W. (2013). Comparison of a high-throughput high-content intracellular *Leishmania donovani* assay with an axenic amastigote assay. *Antimicrobial Agents and Chemotherapy 57*, 2913-2922.

Debrabant, A., Joshi, M.B., Pimenta, P.F.P. and Dwyer, D.M. (2004). Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. *International Journal for Parasitology 34*, 205-217.

Decuypere, S., Rijal, S., Yardley, V., De Doncker, S., Laurent, T., Khanal, B., Chappuis, F. and Dujardin, J.C. (2005). Gene expression analysis of the mechanism of natural Sb(V) resistance in *Leishmania donovani* isolates from Nepal. *Antimicrobial Agents and Chemotherapy 49*, 4616-4621.

Decuypere, S., Vanaerschot, M., Brunker, K., Imamura, H., Muller, S., Khanal, B., Rijal, S., Dujardin, J.C. and Coombs, G.H. (2012). Molecular Mechanisms of Drug Resistance in Natural Leishmania Populations Vary with Genetic Background. *PLoS Neglected Tropical Diseases 6*, e1514.

Delgado, J., Macias, J., Pineda, J.A., Corzo, J.E., Gonzalez-Moreno, M.P., de la Rosa, R., Sanchez-Quijano, A., Leal, M. and Lissen, E. (1999). High frequency of serious side effects from meglumine antimoniate given without an upper limit dose for the treatment of visceral leishmaniasis in human immunodeficiency virus type-1-infected patients. *American Journal of Tropical Medicine and Hygiene 61*, 766-769.

den Boer, M.L., Alvar, J., Davidson, R.N., Ritmeijer, K. and Balasegaram, M. (2009). Developments in the treatment of visceral leishmaniasis. *Expert Opinion on Emerging Drugs 14*, 395-410.

Denton, H., McGregor, J.C. and Coombs, G.H. (2004). Reduction of antileishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. *Biochemical Journal 38*, 405-412.

Deshingkar, P., Kumar, S., Chobey, H.K. and Kumar, D. (2006). The Role of Migration and Remittances in Promoting Livelihoods in Bihar. 1-48.

Desjeux, P., Ghosh, R.S., Dhalaria, P., Strub-Wourgaft, N. and Zijlstra, E.E. (2013). Report of the Post Kala-azar Dermal Leishmaniasis (PKDL) Consortium Meeting, New Delhi, India, 27-29 June 2012. *Parasit. Vectors. 6*, 196.

Detke, S., Katakura, K. and Chang, K.-P. (1989). DNA amplification in arsenite-resistant *Leishmania*. *Experimental Cell Research 180*, 161-170.

Devesa, V., Adair, B.M., Liu, J., Waalkes, M.P., Diwan, B.A., Styblo, M. and Thomas, D.J. (2006). Arsenicals in maternal and fetal mouse tissues after gestational exposure to arsenite. *Toxicology 224*, 147-155.

Dey, S., Dou, D. and Rosen, B.P. (1994). ATP-dependent arsenite transport in everted membrane vesicles of *Escherichia coli*. *Journal of Biological Chemistry 269*, 25442-25446.

Dey, S., Ouellette, M., Lightbody, J., Papadopoulou, B. and Rosen, B.P. (1996). An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. *Proceedings of the National Academy of Sciences of the United States of America 93*, 2192-2197.

Dorlo, T.P.C., Balasegaram, M., Beijnen, J.H. and de Vries, P.J. (2012). Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *Journal of Antimicrobial Chemotherapy 67*, 2576-2597.

Doyle, D. (2009). Notoriety to respectability: a short history of arsenic prior to its present day use in haematology. *British Journal of Haematology 145*, 309-317.

Drobna, Z., Walton, F.S., Paul, D.S., Xing, W., Thomas, D.J. and Styblo, M. (2010). Metabolism of arsenic in human liver: the role of membrane transporters. *Archives of Toxicology 84*, 3-16.

Durand, R., Paul, M., Pratlong, F., Rivollet, D., Dubreuil-Lemaire, M.L., Houin, R., Astier, A. and Deniau, M. (1998). *Leishmania infantum*: lack of parasite resistance to amphotericin B in a clinically resistant visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy 42*, 2141-2143.

Dye, C., Guy, M.W., Elkins, D.B., Wilkes, T.J. and Killick-Kendrick, R. (1987). The life expectancy of phlebotomine sandflies: first field estimates from southern France. *Med. Vet. Entomol. 1*, 417-425.

El Fadili, K., Drummelsmith, J., Roy, G., Jardim, A. and Ouellette, M. (2009). Down regulation of KMP-11 in *Leishmania infantum* axenic antimony resistant amastigotes as revealed by a proteomic screen. *Experimental Parasitology 123*, 51-57.

El Fadili, K., Messier, N., Leprohon, P., Roy, G., Guimond, C., Trudel, N., Saravia, N.G., Pavadopoulou, B., Legare, D. and Ouellette, M. (2005). Role of the ABC transporter MRPA (PGPA) in antimony resistance in *Leishmania infantum* axenic and intracellular amastigotes. *Antimicrobial Agents and Chemotherapy 49*, 1988-1993.

Engstrom, K.S., Hossain, M.B., Lauss, M., Ahmed, S., Raqib, R., Vahter, M. and Broberg, K. (2013). Efficient arsenic metabolism--the AS3MT haplotype is associated with DNA methylation and expression of multiple genes around AS3MT. *Plos One 8*, e53732.

Ephros, M., Waldman, E. and Zilberstein, D. (1997). Pentostam induces resistance to antimony and the preservative chlorocresol in *Leishmania donovani* promastigotes and axenically grown amastigotes. *Antimicrobial Agents and Chemotherapy 41*, 1064-1068.

Eppell, B.A., Newell, A.M. and Brown, E.J. (1989). Adenosine receptors are expressed during differentiation of monocytes to macrophages in vitro. Implications for regulation of phagocytosis. *Journal of Immunology 143*, 4141-4145.

Erickson, M.L. and Barnes, R.J. (2006). Arsenic concentration variability in public water system wells in Minnesota, USA. *Applied Geochemistry 21*, 305-317.

Escobar, J., Varela-Nallar, L., Coddou, C., Nelson, P., Maisey, K., Valdes, D., Aspee, A., Espinosa, V., Rozas, C., Montoya, M., Mandiola, C., Rodriguez, F.E., Acuna-Castillo, C., Escobar, A., Fernandez, R., Diaz, H., Sandoval, M., Imarai, M. and Rios, M. (2010). Oxidative damage in lymphocytes of copper smelter workers correlated to higher levels of excreted arsenic. *Mediators. Inflamm. 2010*, 403830.

Escobar, P., Yardley, V. and Croft, S.L. (2001). Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient scid mice. *Antimicrobial Agents and Chemotherapy 45*, 1872-1875.

Escudero-Lourdes, C., Martinez, F.D., Medina de la Garza CE and Calvillo, R.Q. (2005). Effect of oral chronic intoxication with sodium arsenite on murine giardiasis. *Proc. West Pharmacol. Soc. 48*, 92-99.

Fairlamb, A.H., Blackburn, P., Ulrich, P., Chait, B.T. and Cerami, A. (1985). Trypanothione: a novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. *Science 227*, 1485-1487.

Fakiola, M., Strange, A., Cordell, H.J., Miller, E.N., Pirinen, M., Su, Z., Mishra, A., Mehrotra, S., Monteiro, G.R., Band, G., Bellenguez, C., Dronov, S., Edkins, S., Freeman, C., Giannoulatou, E., Gray, E., Hunt, S.E., Lacerda, H.G., Langford, C., Pearson, R., Pontes, N.N., Rai, M., Singh, S.P., Smith, L., Sousa, O., Vukcevic, D., Bramon, E., Brown, M.A., Casas, J.P., Corvin, A., Duncanson, A., Jankowski, J., Markus, H.S., Mathew, C.G., Palmer, C.N., Plomin, R., Rautanen, A., Sawcer, S.J., Trembath, R.C., Viswanathan, A.C., Wood, N.W., Wilson, M.E., Deloukas, P., Peltonen, L., Christiansen, F., Witt, C., Jeronimo, S.M., Sundar, S., Spencer, C.C., Blackwell, J.M. and Donnelly, P. (2013). Common variants in the HLA-DRB1-HLA-DQA1 HLA class II region are associated with susceptibility to visceral leishmaniasis. *Nature Genetics 45*, 208-213.

Faraut-Gambarelli, F., Piarroux, R., Deniau, M., Giusiano, B., Marty, P., Michel, G., Faugere, B. and Dumon, H. (1997). In vitro and in vivo resistance of *Leishmania infantum* to meglumine antimoniate: A study of 37 strains collected from patients with visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy 41*, 827-830.

Feinglass, E.J. (1973). Arsenic intoxication from well water in the United States. *New England Journal of Medicine 288*, 828-830.

Fernandez, M.M., Malchiodi, E.L. and Algranati, I.D. (2011). Differential effects of paromomycin on ribosomes of *Leishmania mexicana* and mammalian cells. *Antimicrobial Agents and Chemotherapy 55*, 86-93.

Ferreira, C.D., Martins, P.S., Demicheli, C., Brochu, C., Ouellette, M. and Frezerd, F. (2003). Thiol-induced reduction of antimony(V) into antimony(III): A comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione. *Biometals 16*, 441-446.

Filella, M., Belzile, N. and Chen, Y.W. (2002). Antimony in the environment: a review focused on natural waters I. Occurrence. *Earth-Science Reviews 57*, 125-176.

Frezard, F., Demicheli, C., Ferreira, C.S. and Costa, M.A.P. (2001). Glutathione-induced conversion of pentavalent antimony to trivalent antimony in meglumine antimoniate. *Antimicrobial Agents and Chemotherapy 45*, 913-916.

Friedheim, E.A. (1951). Mel B in the treatment of tryparsamide resistant *T. gambiense* sleeping sickness: observations on drug resistance in the trypanosomes of the French Cameroun. *American Journal of Tropical Medicine and Hygiene 31*, 218-235.

Friedheim, E.A.H. (1949). Mel B in the treatment of Human Trypanosomiasis. *American Journal of Tropical Medicine and Hygiene 29*, 173-180.

Friedrich, K., Vieira, F.A., Porrozzi, R., Marchevsky, R.S., Miekeley, N., Grimaldi, G., Jr. and Paumgartten, F.J. (2012). Disposition of antimony in rhesus monkeys infected with *Leishmania braziliensis* and treated with meglumine antimoniate. *Journal of toxicology and environmental health. Part A. 75*, 63-75.

Frith, J. (2013). Arsenic -the "Poison of Kings" and the "Saviour of Syphilis". *Journal of Military and Veterans' Health 21*, 11-17.

Fyfe, P.K., Westrop, G.D., Silva, A.M., Coombs, G.H. and Hunter, W.N. (2012). *Leishmania* TDR1 structure, a unique trimeric glutathione transferase capable of deglutathionylation and antimonial prodrug activation. *Proceedings of the National Academy of Sciences of the United States of America 109*, 11693-11698.

Gamble, M.V. and Liu, X. (2005). Urinary creatinine and arsenic metabolism. *Environmental Health Perspectives 113*, A442-A443.

Garcia-Hernandez, R., Manzano, J.I., Castanys, S. and Gamarro, F. (2012). *Leishmania donovani* develops resistance to drug combinations. *PLoS Neglected Tropical Diseases 6*, e1974.

Gautam, S., Kumar, R., Maurya, R., Nylen, S., Ansari, N., Rai, M., Sundar, S. and Sacks, D. (2011). IL-10 neutralization promotes parasite clearance in splenic aspirate

cells from patients with visceral leishmaniasis. *Journal of Infectious Diseases 204*, 1134-1137.

Gazzard, B. (2001). Tuberculosis, HIV and the developing world. *Clin. Med. 1*, 62-68.

Gebel, T. (1997). Arsenic and antimony: comparative approach on mechanistic toxicology. *Chemico-Biological Interactions 107*, 131-144.

Glaser, T.A., Wells, S.J., Spithill, T.W., Pettitt, J.M., Humphris, D.C. and Mukkada, A.J. (1990). *Leishmania major* and *L. donovani*: A method for rapid purification of amastigotes. *Experimental Parasitology 71*, 343-345.

Gonzalez-Mejia, M.E. and Doseff, A.I. (2009). Regulation of monocytes and macrophages cell fate. *Front Biosci. (Landmark. Ed) 14*, 2413-2431.

Goodwin, L.G. (1995). Pentostam(R) (sodium stibogluconate); a 50-year personal reminiscence. *Transactions of the Royal Society of Tropical Medicine and Hygiene 89*, 339-341.

Gourbal, B., Sonuc, N., Bhattacharjee, H., Legare, D., Sundar, S., Ouellette, M., Rosen, B.P. and Mukhopadhyay, R. (2004). Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin. *Journal of Biological Chemistry 279*, 31010-31017.

Goyard, S., Segawa, H., Gordon, J., Showalter, M., Duncan, R., Turco, S.J. and Beverley, S.M. (2003). An *in vitro* system for developmental and genetic studies of *Leishmania donovani* phosphoglycans. *Molecular and Biochemical Parasitology 130*, 31-42.

Gramiccia, M., Gradoni, L. and Orsini, S. (1992). Decreased sensitivity to meglumine antimoniate (Glucantime) of *Leishmania infantum* isolated from dogs after several courses of drug treatment. *Annals of Tropical Medicine and Parasitology 86*, 613-620.

Grogl, M., Oduola, A.M.J., Cordero, L.D. and Kyle, D.E. (1989). *Leishmania spp*.: development of pentostam-resistant clones in vitro by discontinuous drug exposure. *Experimental Parasitology 69*, 78-90.

Grondin, K., Haimeur, A., Mukhopadhyay, R., Rosen, B.P. and Ouellette, M. (1997). Co-amplification of the g-glutamylcysteine synthetase gene *gsh1* and of the ABC transporter gene *pgpA* in arsenite-resistant *Leishmania tarentolae*. *EMBO Journal 16*, 3057-3065.

Guha Mazumder, D.N., Haque, R., Ghosh, N., De, B.K., Santra, A., Chakraborty, D. and Smith, A.H. (1998). Arsenic levels in drinking water and the prevalence of skin lesions in West Bengal, India. *Int. J. Epidemiol. 27*, 871-877.

Guimond, C., Trudel, N., Brochu, C., Marquis, N., El Fadili, A., Peytavi, R., Briand, G., Richard, D., Messier, N., Papadopoulou, B., Corbeil, J., Bergeron, M.G., Legare, D. and Ouellette, M. (2003). Modulation of gene expression in *Leishmania* drug resistant mutants as determined by targeted DNA microarrays. *Nucleic Acids Research 31*, 5886-5896.

Haimeur, A., Brochu, C., Genest, P.A., Papadopoulou, B. and Ouellette, M. (2000). Amplification of the ABC transporter gene PGPA and increased trypanothione levels in

potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. *Molecular and Biochemical Parasitology 108*, 131-135.

Haimeur, A., Guimond, C., Pilote, S., Mukhopadhyay, R., Rosen, B.P., Poulin, R. and Ouellette, M. (1999). Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant *Leishmania*. *Molecular Microbiology 34*, 726-735.

Haldar, A.K., Yadav, V., Singhal, E., Bisht, K.K., Singh, A., Bhaumik, S., Basu, R., Sen, P. and Roy, S. (2010). *Leishmania donovani* isolates with antimony-resistant but not -sensitive phenotype inhibit sodium antimony gluconate-induced dendritic cell activation. *PLoS Pathogens 6*, e1000907.

Hands, K.J., Cuchet-Lourenco, D., Everett, R.D. and Hay, R.T. (2014). PML isoforms in response to arsenic: high-resolution analysis of PML body structure and degradation. *Journal of Cell Science 127*, 365-375.

Hansen, C., Hansen, E.W., Hansen, H.R., Gammelgaard, B. and Sturup, S. (2011). Reduction of Sb(V) in a human macrophage cell line measured by HPLC-ICP-MS. *Biological Trace Element Research 144*, 234-243.

Hasker, E., Kansal, S., Malaviya, P., Gidwani, K., Picado, A., Singh, R.P., Chourasia, A., Singh, A.K., Shankar, R., Menten, J., Wilson, M.E., Boelaert, M. and Sundar, S. (2013). Latent infection with *Leishmania donovani* in highly endemic villages in Bihar, India. *PLoS Neglected Tropical Diseases 7*, e2053.

Hasker, E., Malaviya, P., Gidwani, K., Picado, A., Ostyn, B., Kansal, S., Singh, R.P., Singh, O.P., Chourasia, A., Kumar, S.A., Shankar, R., Wilson, M.E., Khanal, B., Rijal, S., Boelaert, M. and Sundar, S. (2014). Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and Nepal. *PLoS Neglected Tropical Diseases 8*, e2657.

Hasker, E., Singh, S.P., Malaviya, P., Singh, R.P., Shankar, R., Boelaert, M. and Sundar, S. (2010). Management of visceral leishmaniasis in rural primary health care services in Bihar, India. *Tropical Medicine and International Health 15*, 55-62.

Hassan, E. (2005). Recall bias can be a threat to retrospective and prospective research designs. *The Internet Journal of Epidemiology 3 (2) https://ispub.com/IJE/3/2/13060.*

Hassan, M.M., Atkins, P.J. and Dunn, C.E. (2005). Social implications of arsenic poisoning in Bangladesh. *Soc. Sci. Med. 61*, 2201-2211.

Heath, S., Chance, M.L. and New, R.R. (1984). Quantitative and ultrastructural studies on the uptake of drug loaded liposomes by mononuclear phagocytes infected with *Leishmania donovani*. *Mol. Biochem. Parasitol. 12*, 49-60.

Hegedus, C.M., Skibola, C.F., Warner, M., Skibola, D.R., Alexander, D., Lim, S., Dangleben, N.L., Zhang, L., Clark, M., Pfeiffer, R.M., Steinmaus, C., Smith, A.H., Smith, M.T. and Moore, L.E. (2008). Decreased urinary beta-defensin-1 expression as a biomarker of response to arsenic. *Toxicol. Sci. 106*, 74-82.

Hendrickx, S., Boulet, G., Mondelaers, A., Dujardin, J.C., Rijal, S., Lachaud, L., Cos, P., Delputte, P. and Maes, L. (2014). Experimental selection of paromomycin and

miltefosine resistance in intracellular amastigotes of *Leishmania donovani* and *L. infantum*. *Parasitology Research 113*, 1875-1881.

Hendrickx, S., Da Luz, R.A.I., Bhandari, V., Kuypers, K., Shaw, C.D., Lonchamp, J., Salotra, P., Carter, K., Sundar, S., Rijal, S., Dujardin, J.C., Cos, P. and Maes, L. (2012). Experimental induction of paromomycin resistance in antimony-resistant strains of *L. donovani*: outcome dependent on in vitro selection protocol. *PLoS Neglected Tropical Diseases 6*, e1664.

Herwaldt, B.L. (1999). Leishmaniasis. *Lancet 354*, 1191-1199.

Hess, K.R. (1995). Graphical methods for assessing violations of the proportional hazards assumption in Cox regression. *Statistics in medicine 14*, 1707-1723.

Hira-Smith, M.M., Yuan, Y., Savarimuthu, X., Liaw, J., Hira, A., Green, C., Hore, T., Chakraborty, P., von Ehrenstein, O.S. and Smith, A.H. (2007). Arsenic concentrations and bacterial contamination in a pilot shallow dugwell program in West Bengal, India. *J. Environ. Sci. Health A Tox. Hazard. Subst. Environ. Eng 42*, 89-95.

Ho, P.C. (2005). $_{33}$As Metallotherapeutic Arsenic Compounds. In *Metallotherapeutic Drugs & Metal-based Diagnostic Agents, The use of metals in medicine*, M. Gielen and E.R.T. Tiekink, eds., pp. 297-331.

Holzmuller, P., Sereno, D. and Lemesre, J.L. (2005). Lower nitric oxide susceptibility of trivalent antimony-resistant amastigotes of *Leishmania infantum*. *Antimicrobial Agents and Chemotherapy 49*, 4406-4409.

Hu, L.Q. and Colman, R.F. (1995). Monobromobimane as an affinity label of the xenobiotic binding site of rat glutathione-S-transferase-3-3. *Journal of Biological Chemistry 270*, 21875-21883.

Hurwitz, I., Hillesland, H., Fieck, A., Das, P. and Durvasula, R. (2011). The paratransgenic sand fly: a platform for control of *Leishmania* transmission. *Parasit. Vectors. 4*, 82.

Ivens, A.C., Peacock, C.S., Worthey, E.A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M.A., Adlem, E., Aert, R., Anupama, A., Apostolou, Z., Attipoe, P., Bason, N., Bauser, C., Beck, A., Beverley, S.M., Bianchettin, G., Borzym, K., Bothe, G., Bruschi, C.V., Collins, M., Cadag, E., Ciarloni, L., Clayton, C., Coulson, R.M., Cronin, A., Cruz, A.K., Davies, R.M., De Gaudenzi, J., Dobson, D.E., Duesterhoeft, A., Fazelina, G., Fosker, N., Frasch, A.C., Fraser, A., Fuchs, M., Gabel, C., Goble, A., Goffeau, A., Harris, D., Hertz-Fowler, C., Hilbert, H., Horn, D., Huang, Y., Klages, S., Knights, A., Kube, M., Larke, N., Litvin, L., Lord, A., Louie, T., Marra, M., Masuy, D., Matthews, K., Michaeli, S., Mottram, J.C., Muller-Auer, S., Munden, H., Nelson, S., Norbertczak, H., Oliver, K., O'neil, S., Pentony, M., Pohl, T.M., Price, C., Purnelle, B., Quail, M.A., Rabbinowitsch, E., Reinhardt, R., Rieger, M., Rinta, J., Robben, J., Robertson, L., Ruiz, J.C., Rutter, S., Saunders, D., Schafer, M., Schein, J., Schwartz, D.C., Seeger, K., Seyler, A., Sharp, S., Shin, H., Sivam, D., Squares, R., Squares, S., Tosato, V., Vogt, C., Volckaert, G., Wambutt, R., Warren, T., Wedler, H., Woodward, J., Zhou, S., Zimmermann, W., Smith, D.F., Blackwell, J.M., Stuart, K.D., Barrell, B. and Myler, P.J. (2005). The genome of the kinetoplastid parasite, *Leishmania major*. *Science 309*, 436-442.

Jayatilake, N., Mendis, S., Maheepala, P. and Mehta, F.R. (2013). Chronic kidney disease of uncertain aetiology: prevalence and causative factors in a developing country. *BMC Nephrol. 14*, 180.

Jones, D.C., Hallyburton, I., Stojanovski, L., Read, K.D., Frearson, J.A. and Fairlamb, A.H. (2010). Identification of a kappa-opioid agonist as a potent and selective lead for drug development against human African trypanosomiasis. *Biochemical Pharmacology 80*, 1478-1486.

Kaur, G. and Rajput, B. (2014). Comparative analysis of the omics technologies used to study antimonial, amphotericin B, and pentamidine resistance in Leishmania. *Plos One 2014*, e726328.

Kaye, P.M. and Aebischer, T. (2011). Visceral leishmaniasis: immunology and prospects for a vaccine. *Clin. Microbiol. Infect. 17*, 1462-1470.

Kennedy, P.G. (2013). Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *Lancet Neurol. 12*, 186-194.

Kepler, T.B. and Perelson, A.S. (1998). Drug concentration heterogeneity facilitates the evolution of drug resistance. *Proceedings of the National Academy of Sciences of the United States of America 95*, 11514-11519.

Kesari, S., Bhunia, G.S., Kumar, V., Jeyaram, A., Ranjan, A. and Das, P. (2011). A comparative evaluation of end-emic and non-endemic region of visceral leishmaniasis (Kala-azar) in India with ground survey and space technology. *Memorias do Instituto Oswaldo Cruz 106*, 515-523.

Kitchin, K.T. and Conolly, R. (2010). Arsenic-induced carcinogenesis--oxidative stress as a possible mode of action and future research needs for more biologically based risk assessment. *Chemical Research in Toxicology 23*, 327-335.

Kojima, C., Ramirez, D.C., Tokar, E.J., Himeno, S., Drobna, Z., Styblo, M., Mason, R.P. and Waalkes, M.P. (2009). Requirement of arsenic biomethylation for oxidative DNA damage. *Journal of the National Cancer Institute 101*, 1670-1681.

Kosnett, M.J. (2013). The role of chelation in the treatment of arsenic and mercury poisoning. *Journal of medical toxicology : official journal of the American College of Medical Toxicology 9*, 347-354.

Kozul, C.D., Ely, K.H., Enelow, R.I. and Hamilton, J.W. (2009). Low-dose arsenic compromises the immune response to influenza A infection in vivo. *Environmental Health Perspectives 117*, 1441-1447.

Kritharis, A., Bradley, T.P. and Budman, D.R. (2013). The evolving use of arsenic in pharmacotherapy of malignant disease. *Ann. Hematol. 92*, 719-730.

Kuepfer, I., Schmid, C., Allan, M., Edielu, A., Haary, E.P., Kakembo, A., Kibona, S., Blum, J. and Burri, C. (2012). Safety and efficacy of the 10-day melarsoprol schedule for the treatment of second stage Rhodesiense sleeping sickness. *PLoS Neglected Tropical Diseases 6*, e1695.

Lane, R.P. (1993). Sandflies (Phlebotominae). In *Medical Insects and Arachnids*, R.P. Lane and R.W. Crosskey, eds. (London: Chapman & Hall), pp. 78-119.

Légaré, D., Papadopoulou, B., Roy, G., Mukhopadhyay, R., Haimeur, A., Dey, S., Grondin, K., Brochu, C., Rosen, B.P. and Ouellette, M. (1997). Efflux systems and increased trypanothione levels in arsenite-resistant *Leishmania*. *Experimental Parasitology 87*, 275-282.

Légaré, D., Richard, D., Mukhopadhyay, R., Stierhof, Y.D., Rosen, B.P., Haimeur, A., Papadopoulou, B. and Ouellette, M. (2001). The *Leishmania* ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. *Journal of Biological Chemistry 276*, 26301-26307.

Leprohon, P., Legare, D., Raymond, F., Madore, E., Hardiman, G., Corbeil, J. and Ouellette, M. (2009). Gene expression modulation is associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-resistant Leishmania infantum. *Nucleic Acids Research 37*, 1387-1399.

Lin, S., Shi, Q., Nix, F.B., Styblo, M., Beck, M.A., Herbin-Davist, K.M., Hall, L.L., Simeonsson, J.B. and Thomas, D.J. (2002). A novel *S*-adenosyl-L-methionine : arsenic(III) methyltransferase from rat liver cytosol. *Journal of Biological Chemistry 277*, 10795-10803.

Lin, Y.C., Hsu, J.Y., Chiang, S.C. and Lee, S.T. (2005). Distinct overexpression of cytosolic and mitochondrial tryparedoxin peroxidases results in preferential detoxification of different oxidants in arsenite-resistant *Leishmania amazonensis* with and without DNA amplification. *Molecular and Biochemical Parasitology 142*, 66-75.

Lin, Y.C., Hsu, J.Y., Jui-Hsu, S., Chi, Y., Chiang, S.C. and Lee, S.T. (2008). Two distinct arsenite-resistant variants of *Leishmania amazonensis* take different routes to achieve resistance as revealed by comparative transcriptomics. *Molecular and Biochemical Parasitology 162*, 16-31.

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E. and Sacks, D. (1999). Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of Leishmania donovani. *Journal of Infectious Diseases 180*, 564-567.

Llanos-Cuentas, A., Tulliano, G., Araujo-Castillo, R., Miranda-Verastegui, C., Santamaria-Castrellon, G., Ramirez, L., Lazo, M., De, D.S., Boelaert, M., Robays, J., Dujardin, J.C., Arevalo, J. and Chappuis, F. (2008). Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. *Clinical Infectious Diseases 46*, 223-231.

Lowney, Y.W., Ruby, M.V., Wester, R.C., Schoof, R.A., Holm, S.E., Hui, X.Y., Barbadillo, S. and Maibach, H.I. (2005). Percutaneous absorption of arsenic from environmental media. *Toxicology and Industrial Health 21*, 1-14.

Lu, K., Mahbub, R., Cable, P.H., Ru, H., Parry, N.M., Bodnar, W.M., Wishnok, J.S., Styblo, M., Swenberg, J.A., Fox, J.G. and Tannenbaum, S.R. (2014). Gut microbiome phenotypes driven by host genetics affect arsenic metabolism. *Chemical Research in Toxicology 27*, 172-174.

Lukes, J., Mauricio, I.L., Schonian, G., Dujardin, J.C., Soteriadou, K., Dedet, J.P., Kuhls, K., Tintaya, K.W., Jirku, M., Chocholova, E., Haralambous, C., Pratlong, F., Obornik, M., Horak, A., Ayala, F.J. and Miles, M.A. (2007). Evolutionary and

geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc. Natl. Acad. Sci. U. S. A 104*, 9375-9380.

Madalosso, G., Fortaleza, C.M., Ribeiro, A.F., Cruz, L.L., Nogueira, P.A. and Lindoso, J.A. (2012). American visceral leishmaniasis: factors associated with lethality in the state of São Paulo, Brazil. *J. Trop. Med. 2012*, 281572.

Maes, L., Cos, P., and Croft, S.L. (2013). Drug Resistance in Leishmania Parasites., A.Ponte-Sucre, A.Diaz, and M.Padron-Nieves, eds. (Wien: Springer-Verlag), pp. 407-429.

Maharjan, M., Singh, S., Chatterjee, M. and Madhubala, R. (2008). Role of aquaglyceroporin (AQP1) gene and drug uptake in antimony-resistant clinical isolates of *Leishmania donovani*. *American Journal of Tropical Medicine and Hygiene 79*, 69-75.

Malaviya, P., Picado, A., Singh, S.P., Hasker, E., Singh, R.P., Boelaert, M. and Sundar, S. (2011). Visceral leishmaniasis in Muzaffarpur district, Bihar, India from 1990 to 2008. *Plos One 6*, e14751.

Malhotra-Kumar, S., Lammens, C., Coenen, S., Van, H.K. and Goossens, H. (2007). Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. *Lancet 369*, 482-490.

Mandal, B.K. and Suzuki, K.T. (2002). Arsenic round the world: a review. *Talanta 58*, 201-235.

Mandal, G., Wyllie, S., Singh, N., Sundar, S., Fairlamb, A.H. and Chatterjee, M. (2007). Increased levels of thiols protect antimony unresponsive *Leishmania donovani* field isolates against reactive oxygen species generated by trivalent antimony. *Parasitology 134*, 1679-1687.

Mandal, S., Maharjan, M., Singh, S., Chatterjee, M. and Madhubala, R. (2010). Assessing aquaglyceroporin gene status and expression profile in antimony-susceptible and -resistant clinical isolates of *Leishmania donovani* from India. *Journal of Antimicrobial Chemotherapy 65*, 496-507.

Mascari, T.M., Hanafi, H.A., Jackson, R.E., Ouahabi, S., Ameur, B., Faraj, C., Obenauer, P.J., Diclaro, J.W. and Foil, L.D. (2013). Ecological and control techniques for sand flies (Diptera: Psychodidae) associated with rodent reservoirs of leishmaniasis. *PLoS Neglected Tropical Diseases 7*, e2434.

Matlashewski, G., Arana, B., Kroeger, A., Battacharya, S., Sundar, S., Das, P., Sinha, P.K., Rijal, S., Mondal, D., Zilberstein, D. and Alvar, J. (2011). Visceral leishmaniasis: elimination with existing interventions. *Lancet Infectious Diseases 11*, 322-325.

Mazumder, D.N. (2001). Clinical aspects of chronic arsenic toxicity. *J. Assoc. Physicians India 49*, 650-655.

Mazumder, D.N.G. (2005). Effect of chronic intake of arsenic-contaminated water on liver. *Toxicology and Applied Pharmacology 206*, 169-175.

Mbongo, N., Loiseau, P.M., Billion, M.A. and Robert-Gero, M. (1998). Mechanism of amphotericin B resistance in *Leishmania donovani* promastigotes. *Antimicrobial Agents and Chemotherapy 42*, 352-357.

McCallum, R.I. (1977). President's address. Observations upon antimony. *Proceedings of the Royal Society of Medicine 70*, 756-763.

McCarty, K.M., Senn, D.B., Kile, M.L., Quamruzzaman, Q., Rahman, M., Mahiuddin, G. and Christiani, D.C. (2004). Antimony: An unlikely confounder in the relationship between well water arsenic and health outcomes in Bangladesh. *Environmental Health Perspectives 112*, 809-811.

McDowell, M.A. and Sacks, D.L. (1999). Inhibition of host cell signal transduction by *Leishmania*: observations relevant to the selective impairment of IL-12 responses. *Current Opinion In Microbiology 2*, 438-443.

Meharg, A.A. (2005). Venomous Earth: How Arsenic Caused The World's Worst Mass Poisoning. (New York: Palgrave Macmillan), pp. 1-208.

Melak, D., Ferreccio, C., Kalman, D., Parra, R., Acevedo, J., Perez, L., Cortes, S., Smith, A.H., Yuan, Y., Liaw, J. and Steinmaus, C. (2014). Arsenic methylation and lung and bladder cancer in a case-control study in northern Chile. *Toxicology and Applied Pharmacology 274*, 225-231.

Mishra, M., Biswas, U.K., Jha, A.M. and Khan, A.B. (1994). Amphotericin versus sodium stibogluconate in first-line treatment of Indian kala-azar. *Lancet 344*, 1599-1600.

Mitra, S.R., Mazumder, D.N.G., Basu, A., Block, G., Haque, R., Samanta, S., Ghosh, N., Smith, M.M.H., von Ehrenstein, O.S. and Smith, A.H. (2004). Nutritional factors and susceptibility to arsenic-caused skin lesions in West Bengal, India. *Environmental Health Perspectives 112*, 1104-1109.

Mittal, M.K., Rai, S., Ravinder, A., Gupta, S., Sundar, S. and Goyal, N. (2007). Characterization of natural antimony resistance in *Leishmania donovani* isolates. *American Journal of Tropical Medicine and Hygiene 76*, 681-688.

Mogi, M., Kinpara, K., Kondo, A. and Togari, A. (1999). Involvement of nitric oxide and biopterin in proinflammatory cytokine-induced apoptotic cell death in mouse osteoblastic cell line MC3T3-E1. *Biochemical Pharmacology 58*, 649-654.

Mohamed, H.S., Ibrahim, M.E., Miller, E.N., White, J.K., Cordell, H.J., Howson, J.M., Peacock, C.S., Khalil, E.A., el Hassan, A.M. and Blackwell, J.M. (2004). SLC11A1 (formerly NRAMP1) and susceptibility to visceral leishmaniasis in The Sudan. *Eur. J. Hum. Genet. 12*, 66-74.

Mondal, D., Singh, S.P., Kumar, N., Joshi, A., Sundar, S., Das, P., Siddhivinayak, H., Kroeger, A. and Boelaert, M. (2009). Visceral Leishmaniasis elimination programme in India, Bangladesh, and Nepal: reshaping the case finding/case management strategy. *PLoS Neglected Tropical Diseases 3*, e355.

Monnet, D.L. (2000). [Antibiotic use and bacterial resistance]. *Ann. Fr. Anesth. Reanim. 19*, 409-417.

Mookerjee, B.J., Mookerjee, A., Banerjee, R., Saha, M., Singh, S., Naskar, K., Tripathy, G., Sinha, P.K., Pandey, K., Sundar, S., Bimal, S., Das, P.K., Choudhuri, S.K. and Roy, S. (2008). Inhibition of ABC transporters abolishes antimony resistance in *Leishmania* Infection. *Antimicrobial Agents and Chemotherapy 52*, 1080-1093.

Mookerjee, B.J., Mookerjee, A., Sen, P., Bhaumik, S., Sen, P., Banerjee, S., Naskar, K., Choudhuri, S.K., Saha, B., Raha, S. and Roy, S. (2006). Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. *Antimicrobial Agents and Chemotherapy 50*, 1788-1797.

Mueller, Y., Mbulamberi, D.B., Odermatt, P., Hoffmann, A., Loutan, L. and Chappuis, F. (2009). Risk factors for in-hospital mortality of visceral leishmaniasis patients in eastern Uganda. *Tropical Medicine and International Health 14*, 910-917.

Mukherjee, A., Padmanabhan, P.K., Singh, S., Roy, G., Girard, I., Chatterjee, M., Ouellette, M. and Madhubala, R. (2007). Role of ABC transporter MRPA, g-glutamylcysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of *Leishmania donovani*. *Journal of Antimicrobial Chemotherapy 59*, 204-211.

Mukhopadhyay, R., Dey, S., Xu, N., Gage, D., Lightbody, J., Ouellette, M. and Rosen, B.P. (1996). Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. *Proceedings of the National Academy of Sciences of the United States of America 93*, 10383-10387.

Mukhopadhyay, R., Mukherjee, S., Mukherjee, B., Naskar, K., Mondal, D., Decuypere, S., Ostyn, B., Prajapati, V.K., Sundar, S., Dujardin, J.C. and Roy, S. (2011). Characterisation of antimony-resistant *Leishmania donovani* isolates: biochemical and biophysical studies and interaction with host cells. *International Journal for Parasitology 41*, 1311-1321.

Mukhopadhyay, R., Rosen, B.P., Pung, L.T. and Silver, S. (2002). Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiology Reviews 26*, 311-325.

Murray, H.W. (2005). Interleukin 10 receptor blockade--pentavalent antimony treatment in experimental visceral leishmaniasis. *Acta Tropica 93*, 295-301.

Murray, H.W., Berman, J.D., Davies, C.R. and Saravia, N.G. (2005). Advances in leishmaniasis. *Lancet 366*, 1561-1577.

Murray, H.W., Montelibano, C., Peterson, R. and Sypek, J.P. (2000). Interleukin-12 regulates the response to chemotherapy in experimental visceral Leishmaniasis. *Journal of Infectious Diseases 182*, 1497-1502.

Murray, H.W., Oca, M.J., Granger, A.M. and Schreiber, R.D. (1989). Requirement for T cells and effect of lymphokines in successful chemotherapy for an intracellular infection. Experimental visceral leishmaniasis. *Journal of Clinical Investigation 83*, 1253-1257.

Musa, A., Khalil, E., Hailu, A., Olobo, J., Balasegaram, M., Omollo, R., Edwards, T., Rashid, J., Mbui, J., Musa, B., Abuzaid, A.A., Ahmed, O., Fadlalla, A., El-Hassan, A., Mueller, M., Mucee, G., Njoroge, S., Manduku, V., Mutuma, G., Apadet, L., Lodenyo, H., Mutea, D., Kirigi, G., Yifru, S., Mengistu, G., Hurissa, Z., Hailu, W., Weldegebreal,

T., Tafes, H., Mekonnen, Y., Makonnen, E., Ndegwa, S., Sagaki, P., Kimutai, R., Kesusu, J., Owiti, R., Ellis, S. and Wasunna, M. (2012). Sodium stibogluconate (SSG) & paromomycin combination compared to SSG for visceral leishmaniasis in East Africa: a randomised controlled trial. *PLoS Neglected Tropical Diseases 6*, e1674.

New, R.R., Chance, M.L. and Heath, S. (1981). Antileishmanial activity of amphotericin and other antifungal agents entrapped in liposomes. *Journal of Antimicrobial Chemotherapy 8*, 371-381.

Ng, A.W., Wasan, K.M. and Lopez-Berestein, G. (2003). Development of liposomal polyene antibiotics: an historical perspective. *Journal of Pharmacy & Pharmaceutical Sciences 6*, 67-83.

Nickson, R., McArthur, J., Burgess, W., Ahmed, K.M., Ravenscroft, P. and Rahman, M. (1998). Arsenic poisoning of Bangladesh groundwater. *Nature 395*, 338.

Nickson, R., Sengupta, C., Mitra, P., Dave, S.N., Banerjee, A.K., Bhattacharya, A., Basu, S., Kakoti, N., Moorthy, N.S., Wasuja, M., Kumar, M., Mishra, D.S., Ghosh, A., Vaish, D.P., Srivastava, A.K., Tripathi, R.M., Singh, S.N., Prasad, R., Bhattacharya, S. and Deverill, P. (2007). Current knowledge on the distribution of arsenic in groundwater in five states of India. *Journal of Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering 42*, 1707-1718.

Nordstrom, D.K. (2002). Public health. Worldwide occurrences of arsenic in ground water. *Science 296*, 2143-2145.

Olliaro, P.L., Guerin, P.J., Gerstl, S., Haaskjold, A.A., Rottingen, J.A. and Sundar, S. (2005). Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980-2004. *Lancet Infectious Diseases 5*, 763-774.

Oremland, R.S. and Stolz, J.F. (2005). Arsenic, microbes and contaminated aquifers. *Trends in Microbiology 13*, 45-49.

Ostyn, B., Malaviya, P., Hasker, E., Uranw, S., Singh, R.P., Rijal, S., Sundar, S., Dujardin, J.C. and Boelaert, M. (2013). Retrospective quarterly cohort monitoring for patients with visceral leishmaniasis in the Indian subcontinent: outcomes of a pilot project. *Tropical Medicine and International Health 18*, 725-733.

Ostyn, B., Vanlerberghe, V., Picado, A., Dinesh, D.S., Sundar, S., Chappuis, F., Rijal, S., Dujardin, J.C., Coosemans, M., Boelaert, M. and Davies, C. (2008). Vector control by insecticide-treated nets in the fight against visceral leishmaniasis in the Indian subcontinent, what is the evidence? *Tropical Medicine and International Health 13*, 1073-1085.

Park, E.K., Jung, H.S., Yang, H.I., Yoo, M.C., Kim, C. and Kim, K.S. (2007). Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflammation Research 56*, 45-50.

Pathak, M.K. and Yi, T. (2001). Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. *Journal of Immunology 167*, 3391-3397.

Patterson, S., Wyllie, S., Stojanovski, L., Perry, M.R., Simeons, F.R., Norval, S., Osuna-Cabello, M., De, R.M., Read, K.D. and Fairlamb, A.H. (2013). The *R* enantiomer of the anti-tubercular drug PA-824 as a potential oral treatment for visceral leishmaniasis. *Antimicrobial Agents and Chemotherapy 57*, 4699-4706.

Payne, D. (1988). Did medicated salt hasten the spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitology Today 4*, 112-115.

Peacock, C.S., Seeger, K., Harris, D., Murphy, L., Ruiz, J.C., Quail, M.A., Peters, N., Adlem, E., Tivey, A., Aslett, M., Kerhornou, A., Ivens, A., Fraser, A., Rajandream, M.A., Carver, T., Norbertczak, H., Chillingworth, T., Hance, Z., Jagels, K., Moule, S., Ormond, D., Rutter, S., Squares, R., Whitehead, S., Rabbinowitsch, E., Arrowsmith, C., White, B., Thurston, S., Bringaud, F., Baldauf, S.L., Faulconbridge, A., Jeffares, D., Depledge, D.P., Oyola, S.O., Hilley, J.D., Brito, L.O., Tosi, L.R.O., Barrell, B., Cruz, A.K., Mottram, J.C., Smith, D.F. and Berriman, M. (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nature Genetics 39*, 839-847.

Pépin, J., Milord, F., Khonde, A.N., Niyonsenga, T., Loko, L., Mpia, B. and De Wals, P. (1995). Risk factors for encephalopathy and mortality during melarsoprol treatment of *Trypanosoma brucei gambiense* sleeping sickness. *Transactions of the Royal Society of Tropical Medicine and Hygiene 89*, 92-97.

Perry, M.R., Wyllie, S., Prajapati, V.K., Feldmann, J., Sundar, S., Boelaert, M. and Fairlamb, A.H. (2011). Visceral leishmaniasis and arsenic: an ancient poison contributing to antimonial treatment failure in the Indian subcontinent? *PLoS Neglected Tropical Diseases 5*, e1227.

Perry, M.R., Wyllie, S., Raab, A., Feldmann, J. and Fairlamb, A.H. (2013). Chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs in a mouse model of visceral leishmaniasis. *Proceedings of the National Academy of Sciences of the United States of America 110*, 19932-19937.

Pescher, P., Blisnick, T., Bastin, P. and Spath, G.F. (2011). Quantitative proteome profiling informs on phenotypic traits that adapt *Leishmania donovani* for axenic and intracellular proliferation. *Cell Microbiol 13*, 978-991.

Peters, W. (1981). The treatment of kala-azar - new approaches to an old problem. *Indian Journal of Medical Research 73*, 1-18.

Pi, J.B., Kumagai, Y., Sun, G.F., Yamauchi, H., Yoshida, T., Iso, H., Endo, A., Yu, L.Y., Yuki, K.C., Miyauchi, T. and Shimojo, N. (2000). Decreased serum concentrations of nitric oxide metabolites among Chinese in an endemic area of chronic arsenic poisoning in Inner Mongolia. *Free Radical Biology and Medicine 28*, 1137-1142.

Picado, A., Dash, A.P., Bhattacharya, S. and Boelaert, M. (2012). Vector control interventions for visceral leishmaniasis elimination initiative in South Asia, 2005-2010. *Indian Journal of Medical Research 136*, 22-31.

Picado, A., Singh, S.P., Rijal, S., Sundar, S., Ostyn, B., Chappuis, F., Uranw, S., Gidwani, K., Khanal, B., Rai, M., Paudel, I.S., Das, M.L., Kumar, R., Srivastava, P., Dujardin, J.C., Vanlerberghe, V., Andersen, E.W., Davies, C.R. and Boelaert, M.

(2010). Longlasting insecticidal nets for prevention of *Leishmania donovani* infection in India and Nepal: paired cluster randomised trial. *British Medical Journal 341*, c6760.

Price, L.B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P.S., Pearson, T., Waters, A.E., Foster, J.T., Schupp, J., Gillece, J., Driebe, E., Liu, C.M., Springer, B., Zdovc, I., Battisti, A., Franco, A., Zmudzki, J., Schwarz, S., Butaye, P., Jouy, E., Pomba, C., Porrero, M.C., Ruimy, R., Smith, T.C., Robinson, D.A., Weese, J.S., Arriola, C.S., Yu, F., Laurent, F., Keim, P., Skov, R. and Aarestrup, F.M. (2012). Staphylococcus aureus CC398: host adaptation and emergence of methicillin resistance in livestock. *MBio. 3*.

Rahman, A., Persson, L.A., Nermell, B., El, A.S., Ekstrom, E.C., Smith, A.H. and Vahter, M. (2010). Arsenic exposure and risk of spontaneous abortion, stillbirth, and infant mortality. *Epidemiology 21*, 797-804.

Rahman, A., Vahter, M., Ekstrom, E.C. and Persson, L.A. (2011). Arsenic exposure in pregnancy increases the risk of lower respiratory tract infection and diarrhea during infancy in Bangladesh. *Environmental Health Perspectives 119*, 719-724.

Rahman, A., Vahter, M., Ekstrom, E.C., Rahman, M., Golam Mustafa, A.H., Wahed, M.A., Yunus, M. and Persson, L.A. (2007). Association of arsenic exposure during pregnancy with fetal loss and infant death: a cohort study in Bangladesh. *Am. J. Epidemiol. 165*, 1389-1396.

Rai, K., Cuypers, B., Bhattarai, N.R., Uranw, S., Berg, M., Ostyn, B., Dujardin, J.C., Rijal, S. and Vanaerschot, M. (2013a). Relapse after treatment with miltefosine for visceral leishmaniasis is associated with increased infectivity of the infecting *Leishmania donovani* strain. *MBio. 4*, e00611-e00613.

Rai, S., Bhaskar, Goel, S.K., Nath, D.U., Sundar, S. and Goyal, N. (2013b). Role of efflux pumps and intracellular thiols in natural antimony resistant isolates of *Leishmania donovani*. *Plos One 8*, e74862.

Rais, S., Perianin, A., Lenoir, M., Sadak, A., Rivollet, D., Paul, M. and Deniau, M. (2000). Sodium stibogluconate (Pentostam) potentiates oxidant production in murine visceral leishmaniasis and in human blood. *Antimicrobial Agents and Chemotherapy 44*, 2406-2410.

Ravenscroft, P., Brammer, H., and Richards, K. (2009). Arsenic Pollution: A Global Synthesis. (Chichester: Wiley-Blackwell), pp. 1-588.

Ravenscroft, P., McArthur, J.M. and Hoque, M.A. (2013). Stable groundwater quality in deep aquifers of Southern Bangladesh: the case against sustainable abstraction. *Sci. Total Environ. 454-455*, 627-638.

Raymond, F., Boisvert, S., Roy, G., Ritt, J.F., Legare, D., Isnard, A., Stanke, M., Olivier, M., Tremblay, M.J., Papadopoulou, B., Ouellette, M. and Corbeil, J. (2012). Genome sequencing of the lizard parasite *Leishmania tarentolae* reveals loss of genes associated to the intracellular stage of human pathogenic species. *Nucleic Acids Research 40*, 1131-1147.

Reagan-Shaw, S., Nihal, M. and Ahmad, N. (2008). Dose translation from animal to human studies revisited. *FASEB Journal 22*, 659-661.

Reuer, M.K., Bower, N.W., Koball, J.H., Hinostroza, E., de la Torre, M.E., Hurtado, J.A. and Echevarria, S. (2012). Lead, arsenic, and cadmium contamination and its impact on children's health in La Oroya, Peru. *ISRN Public Health 2012*, Article ID 231458.

Rijal, S., Bhandari, S., Koirala, S., Singh, R., Khanal, B., Loutan, L., Dujardin, J.C., Boelaert, M. and Chappuis, F. (2010). Clinical risk factors for therapeutic failure in kala-azar patients treated with pentavalent antimonials in Nepal. *Transactions of the Royal Society of Tropical Medicine and Hygiene 104*, 225-229.

Rijal, S., Yardley, V., Chappuis, F., Decuypere, S., Khanal, B., Singh, R., Boelaert, M., De, D.S., Croft, S. and Dujardin, J.C. (2007). Antimonial treatment of visceral leishmaniasis: are current in vitro susceptibility assays adequate for prognosis of in vivo therapy outcome? *Microbes and Infection 9*, 529-535.

Roberts, W.L., Berman, J.D. and Rainey, P.M. (1995). In vitro antileishmanial properties of tri- and pentavalent antimonial preparations. *Antimicrobial Agents and Chemotherapy 39*, 1234-1239.

Rochette, A., Raymond, F., Corbeil, J., Ouellette, M. and Papadopoulou, B. (2009). Whole-genome comparative RNA expression profiling of axenic and intracellular amastigote forms of *Leishmania infantum*. *Molecular and Biochemical Parasitology 165*, 32-47.

Rogers, M.E. and Bates, P.A. (2007). *Leishmania* manipulation of sand fly feeding behavior results in enhanced transmission. *PLoS Pathogens 3*, e91.

Rogers, M.E., Ilg, T., Nikolaev, A.V., Ferguson, M.A. and Bates, P.A. (2004). Transmission of cutaneous leishmaniasis by sand flies is enhanced by regurgitation of fPPG. *Nature 430*, 463-467.

Rolao, N., Melo, C. and Campino, L. (2004). Influence of the inoculation route in BALB/c mice infected by *Leishmania infantum*. *Acta Tropica 90*, 123-126.

Rosenzweig, D., Smith, D., Opperdoes, F., Stern, S., Olafson, R.W. and Zilberstein, D. (2008). Retooling Leishmania metabolism: from sand fly gut to human macrophage. *FASEB Journal 22*, 590-602.

Ross, R. (1903). Further notes on Leishman's bodies. *British Medical Journal 2*, 1401.

Rossman, T.G. and Klein, C.B. (2011). Genetic and epigenetic effects of environmental arsenicals. *Metallomics. 3*, 1135-1141.

Rubin SS, D.C., Alava, P., Zekker, I., Du, L.G. and Van de Wiele, T. (2014). Arsenic thiolation and the role of sulfate-reducing bacteria from the human intestinal tract. *Environmental Health Perspectives 122*, 817-822.

Sacks, D. and Anderson, C. (2004). Re-examination of the immunosuppressive mechanisms mediating non-cure of *Leishmania* infection in mice. *Immunological Reviews 201*, 225-238.

Sacks, D. and Sher, A. (2002). Evasion of innate immunity by parasitic protozoa. *Nature Immunology 3*, 1041-1047.

Sakurai, T., Ohta, T., Tomita, N., Kojima, C., Hariya, Y., Mizukami, A. and Fujiwara, K. (2004). Evaluation of immunotoxic and immunodisruptive effects of inorganic arsenite on human monocytes/macrophages. *International Immunopharmacology 4*, 1661-1673.

Salgado-Bustamante, M., Ortiz-Perez, M.D., Calderon-Aranda, E., Estrada-Capetillo, L., Nino-Moreno, P., Gonzalez-Amaro, R. and Portales-Perez, D. (2010). Pattern of expression of apoptosis and inflammatory genes in humans exposed to arsenic and/or fluoride. *Sci. Total Environ. 408*, 760-767.

Salih, N.A., van, G.J., Chappuis, F., Antierens, A., Mumina, A., Hammam, O., Boulle, P., Alirol, E., Alnour, M., Elhag, M.S., Manzi, M., Kizito, W. and Zachariah, R. (2014). Liposomal amphotericin B for complicated visceral leishmaniasis (kala-azar) in eastern Sudan: how effective is treatment for this neglected disease? *Tropical Medicine and International Health 19*, 146-152.

Sampaio, M.J., Cavalcanti, N.V., Alves, J.G., Filho, M.J. and Correia, J.B. (2010). Risk factors for death in children with visceral leishmaniasis. *PLoS Neglected Tropical Diseases 4*, e877.

School of Environmental Studies. (2002). Groundwater arsenic contamination in middle Ganga plain, Bihar, India: a future danger? *School of Environmental Studies, Jadavpur University* .

Seifert, K., Escobar, P. and Croft, S.L. (2010). In vitro activity of anti-leishmanial drugs against *Leishmania donovani* is host cell dependent. *Journal of Antimicrobial Chemotherapy 65*, 508-511.

Sekhon, B.S. (2013). Metalloid compounds as drugs. *Research in Pharmaceutical Sciences 8*, 145-158.

Sen, P. and Biswas, T. (2013). Arsenic: the largest mass poisoning of a population in history. *British Medical Journal 346*, f3625.

Sereno, D., Holzmuller, P., Mangot, I., Cuny, G., Ouaissi, A. and Lemesre, J.L. (2001). Antimonial-mediated DNA fragmentation in *Leishmania infantum* amastigotes. *Antimicrobial Agents and Chemotherapy 45*, 2064-2069.

Shaked-Mishan, P., Ulrich, N., Ephros, M. and Zilberstein, D. (2001). Novel intracellular Sb-V reducing activity correlates with antimony susceptibility in *Leishmania donovani*. *Journal of Biological Chemistry 276*, 3971-3976.

Shim, H. and Fairlamb, A.H. (1988). Levels of polyamines, glutathione and glutathione-spermidine conjugates during growth of the insect trypanosomatid *Crithidia fasciculata*. *Journal of General Microbiology 134*, 807-817.

Shimony, O. and Jaffe, C.L. (2008). Rapid fluorescent assay for screening drugs on *Leishmania* amastigotes. *Journal of Microbiological Methods 75*, 196-200.

Singh, N. and Roy, S. (2009). Kala-Azar: An Odyssey into the Past. In *Kala-Azar: An Odyssey into the Past*, (Lucknow: International Book Distributing Co.), pp. 1-28.

Singh, O.P., Hasker, E., Sacks, D., Boelaert, M. and Sundar, S. (2014). Asymptomatic leishmania infection: a new challenge for leishmania control. *Clinical Infectious Diseases 58*, 1424-1429.

Singh, V.P., Ranjan, A., Topno, R.K., Verma, R.B., Siddique, N.A., Ravidas, V.N., Kumar, N., Pandey, K. and Das, P. (2010). Estimation of under-reporting of visceral leishmaniasis cases in Bihar, India. *American Journal of Tropical Medicine and Hygiene 82*, 9-11.

Smith, A.H., Marshall, G., Yuan, Y., Ferreccio, C., Liaw, J., von, E.O., Steinmaus, C., Bates, M.N. and Selvin, S. (2006). Increased mortality from lung cancer and bronchiectasis in young adults after exposure to arsenic in utero and in early childhood. *Environmental Health Perspectives 114*, 1293-1296.

Smith, A.H., Marshall, G., Yuan, Y., Liaw, J., Ferreccio, C. and Steinmaus, C. (2011). Evidence from Chile that arsenic in drinking water may increase mortality from pulmonary tuberculosis. *Am. J. Epidemiol. 173*, 414-420.

Sohel, N., Persson, L.A., Rahman, M., Streatfield, P.K., Yunus, M., Ekstrom, E.C. and Vahter, M. (2009). Arsenic in drinking water and adult mortality: a population-based cohort study in rural Bangladesh. *Epidemiology 20*, 824-830.

Sohlenius-Sternbeck, A.K. (2006). Determination of the hepatocellularity number for human, dog, rabbit, rat and mouse livers from protein concentration measurements. *Toxicology in Vitro 20*, 1582-1586.

Soleman, N., Chandramohan, D. and Shibuya, K. (2006). Verbal autopsy: current practices and challenges. *Bulletin of the World Health Organization 84*, 239-245.

Soto-Pena, G.A., Luna, A.L., Acosta-Saavedra, L., Conde-Moo, P., Lopez-Carrillo, L., Cebrian, M.E., Bastida, M., Calderon-Aranda, E.S. and Vega, L. (2006). Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. *FASEB Journal 20*, 779-+.

Späth, G.F., Garraway, L.A., Turco, S.J. and Beverley, S.M. (2003). The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. *Proceedings of the National Academy of Sciences of the United States of America 100*, 9536-9541.

Spivey, A. (2011). Arsenic and infectious disease: a potential factor in morbidity among Bangladeshi children. *Environmental Health Perspectives 119*, A218.

Srivastava, P., Dayama, A., Mehrotra, S. and Sundar, S. (2011a). Diagnosis of visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene 105*, 1-6.

Srivastava, P., Prajapati, V.K., Rai, M. and Sundar, S. (2011b). Unusual case of resistance to amphotericin B in visceral leishmaniasis in a region in India where leishmaniasis is not endemic. *Journal of Clinical Microbiology 49*, 3088-3091.

States, J.C., Barchowsky, A., Cartwright, I.L., Reichard, J.F., Futscher, B.W. and Lantz, R.C. (2011). Arsenic toxicology: translating between experimental models and human pathology. *Environmental Health Perspectives 119*, 1356-1363.

Stauber, L.A. (1958). Host resistance to the Khartoum strain of *Leishmania donovani*. *The Rice Institute Pamphlet 45*, 80-96.

Stauch, A., Duerr, H.P., Dujardin, J.C., Vanaerschot, M., Sundar, S. and Eichner, M. (2012). Treatment of visceral leishmaniasis: model-based analyses on the spread of antimony-resistant *L. donovani* in Bihar, India. *PLoS Neglected Tropical Diseases 6*, e1973.

Stuart, K.D., Brun, R., Croft, S.L., Fairlamb, A.H., Gurtler, R.E., McKerrow, J.H., Reed, S. and Tarleton, R.L. (2008). Kinetoplastids: related protozoan pathogens, different diseases. *Journal of Clinical Investigation 118*, 1301-1310.

Styblo, M., Del Razo, L.M., Vega, L., Germolec, D.R., LeCluyse, E.L., Hamilton, G.A., Reed, W., Wang, C., Cullen, W.R. and Thomas, D.J. (2000). Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells. *Archives of Toxicology 74*, 289-299.

Subbarayan, P.R. and Ardalan, B. (2014). In the war against solid tumors arsenic trioxide need partners. *J. Gastrointest. Cancer* .

Sudhandiran, G. and Shaha, C. (2003). Antimonial-induced increase in intracellular Ca2+ through non- selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. *Journal of Biological Chemistry 278*, 25120-25132.

Sundar, S. (2001). Drug resistance in Indian visceral leishmaniasis. *Tropical Medicine and International Health 6*, 849-854.

Sundar, S. and Chakravarty, J. (2010). Antimony toxicity. *Int. J. Environ. Res. Public Health 7*, 4267-4277.

Sundar, S., Chakravarty, J., Agarwal, D., Rai, M. and Murray, H.W. (2010). Single-dose liposomal amphotericin B for visceral leishmaniasis in India. *New England Journal of Medicine 362*, 504-512.

Sundar, S., Jha, T.K., Thakur, C.P., Engel, J., Sindermann, H., Fischer, C., Junge, K., Bryceson, A. and Berman, J. (2002). Oral miltefosine for Indian visceral leishmaniasis. *New England Journal of Medicine 347*, 1739-1746.

Sundar, S., Jha, T.K., Thakur, C.P., Sinha, P.K. and Bhattacharya, S.K. (2007). Injectable paromomycin for visceral leishmaniasis in India. *New England Journal of Medicine 356*, 2571-2581.

Sundar, S., Mondal, D., Rijal, S., Bhattacharya, S., Ghalib, H., Kroeger, A., Boelaert, M., Desjeux, P., Richter-Airijoki, H. and Harms, G. (2008). Implementation research to support the initiative on the elimination of kala azar from Bangladesh, India and Nepal - the challenges for diagnosis and treatment. *Tropical Medicine and International Health 13*, 2-5.

Sundar, S., More, D.K., Singh, M.K., Singh, V.P., Sharma, S., Makharia, A., Kumar, P.C.K. and Murray, H.W. (2000). Failure of pentavalent antimony in visceral leishmaniasis in India: Report from the center of the Indian epidemic. *Clinical Infectious Diseases 31*, 1104-1107.

Sundar, S. and Rai, M. (2002). Laboratory diagnosis of visceral leishmaniasis. *Clinical and Diagnostic Laboratory Immunology 9*, 951-958.

Sundar, S., Rosenkaimer, F., Lesser, M.L. and Murray, H.W. (1995). Immunochemotherapy for a systemic intracellular infection: accelerated response using interferon-gamma in visceral leishmaniasis. *Journal of Infectious Diseases 171*, 992-996.

Sundar, S., Singh, A., Rai, M., Prajapati, V.K., Singh, A.K., Ostyn, B., Boelaert, M., Dujardin, J.C. and Chakravarty, J. (2012). Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clinical Infectious Diseases 55*, 543-550.

Sundar, S., Singh, V.P., Sharma, S., Makharia, M.K. and Murray, H.W. (1997). Response to interferon-gamma plus pentavalent antimony in Indian visceral leishmaniasis. *Journal of Infectious Diseases 176*, 1117-1119.

Sundar, S., Sinha, P.R., Agrawal, N.K., Srivastava, R., Rainey, P.M., Berman, J.D., Murray, H.W. and Singh, V.P. (1998). A cluster of cases of severe cardiotoxicity among kala-azar patients treated with a high-osmolarity lot of sodium antimony gluconate. *American Journal of Tropical Medicine and Hygiene 59*, 139-143.

Sundar, S., Thakur, B.B., Tandon, A.K., Agrawal, N.R., Mishra, C.P., Mahapatra, T.M. and Singh, V.P. (1994). Clinicoepidemiological study of drug resistance in Indian kala-azar. *British Medical Journal 308*, 307.

Thakur, C.P. (1984). Epidemiological, clinical and therapeutic features of Bihar kala-azar (including post kala-azar dermal leishmaniasis). *Transactions of the Royal Society of Tropical Medicine and Hygiene 78*, 391-398.

Thakur, C.P., Mitra, D.K. and Narayan, S. (2003). Skewing of cytokine profiles towards T helper cell type 2 response in visceral leishmaniasis patients unresponsive to sodium antimony gluconate. *Transactions of the Royal Society of Tropical Medicine and Hygiene 97*, 409-412.

Thakur, C.P., Narayan, S. and Ranjan, A. (2004). Epidemiological, clinical & pharmacological study of antimony-resistant visceral leishmaniasis in Bihar, India. *Indian Journal of Medical Research 120*, 166-172.

Thomas, H.W. (1905). Some experiments in the treatment of trypanosomiasis. *British Medical Journal 1*, 1140-1143.

Thornton, S.J., Wasan, K.M., Piecuch, A., Lynd, L.L. and Wasan, E.K. (2010). Barriers to treatment for visceral leishmaniasis in hyperendemic areas: India, Bangladesh, Nepal, Brazil and Sudan. *Drug Dev. Ind. Pharm. 36*, 1312-1319.

Torres, D.C., Adaui, V., Ribeiro-Alves, M., Romero, G.A., Arevalo, J., Cupolillo, E. and Dujardin, J.C. (2010). Targeted gene expression profiling in *Leishmania braziliensis* and *Leishmania guyanensis* parasites isolated from Brazilian patients with different antimonial treatment outcomes. *Infect. Genet. Evol. 10*, 727-733.

Tseng, W.P., Chu, H.M., How, S.W., Fong, J.M., Lin, C.S. and Yeh, S. (1968). Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. *Journal of the National Cancer Institute 40*, 453-463.

Tuon, F.F., Neto, V.A. and Amato, V.S. (2008). *Leishmania*: origin, evolution and future since the Precambrian. *FEMS Immunology and Medical Microbiology 54*, 158-166.

Tyler, C.R. and Allan, A.M. (2014). The effects of arsenic exposure on neurological and cognitive dysfunction in human and rodent studies: a review. *Curr. Environ. Health Rep. 1*, 132-147.

Uzcategui, N.L., Zhou, Y., Figarella, K., Ye, J., Mukhopadhyay, R. and Bhattacharjee, H. (2008). Alteration in glycerol and metalloid permeability by a single mutation in the extracellular C-loop of *Leishmania major* aquaglyceroporin LmAQP1. *Molecular Microbiology 70*, 1477-1486.

Vahter, M. (1994). What are the chemical forms of arsenic in urine, and what can they tell us about exposure? *Clinical Chemistry 40*, 679-680.

Vahter, M. (1999). Methylation of inorganic arsenic in different mammalian species and population groups. *Science Progess 82*, 69-88.

Vahter, M. (2002). Mechanisms of arsenic biotransformation. *Toxicology 181-182*, 211-217.

Vahter, M. and Concha, G. (2001). Role of metabolism in arsenic toxicity. *Pharmacology and Toxicology 89*, 1-5.

Valentinus, B. and Kerkringius, T. (1893). The Triumphal Chariot of Antimony (translated from the Latin version published in 1685). (London: James Elliot & Co), pp. 1-204.

Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A. and Laxminarayan, R. (2014). Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infectious Diseases* .

van den Bogaart, E., Berkhout, M.M., Nour, A.B., Mens, P.F., Talha, A.B., Adams, E.R., Ahmed, H.B., Abdelrahman, S.H., Ritmeijer, K., Nour, B.Y. and Schallig, H.D. (2013). Concomitant malaria among visceral leishmaniasis in-patients from Gedarif and Sennar States, Sudan: a retrospective case-control study. *BMC Public Health 13*, e332.

van Griensven J., Balasegaram, M., Meheus, F., Alvar, J., Lynen, L. and Boelaert, M. (2010). Combination therapy for visceral leishmaniasis. *Lancet Infectious Diseases 10*, 184-194.

Vanaerschot, M., De Doncker, S., Rijal, S., Maes, L., Dujardin, J.C. and Decuypere, S. (2011). Antimonial resistance in *Leishmania donovani* is associated with increased *in vivo* parasite burden. *Plos One 6*, e23120.

Vanaerschot, M., Decuypere, S., Berg, M., Roy, S. and Dujardin, J.C. (2013). Drug-resistant microorganisms with a higher fitness - can medicines boost pathogens? *Critical Reviews in Microbiology 39*, 384-394.

Vanaerschot, M., Maes, I., Ouakad, M., Adaui, V., Maes, L., De Doncker, S., Rijal, S., Chappuis, F., Dujardin, J.C. and Decuypere, S. (2010). Linking *in vitro* and *in vivo* survival of clinical *Leishmania donovani* strains. *Plos One 5*, e12211.

Veeken, H., Ritmeijer, K., Seaman, J. and Davidson, R. (2000). A randomized comparison of branded sodium stibogluconate and generic sodium stibogluconate for the treatment of visceral leishmaniasis under field conditions in Sudan. *Tropical Medicine and International Health 5*, 312-317.

Vermeersch, M., da Luz, R.I., Tote, K., Timmermans, J.P., Cos, P. and Maes, L. (2009). In vitro susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrobial Agents and Chemotherapy 53*, 3855-3859.

Wanderley, J.L., Pinto, d.S.L., Deolindo, P., Soong, L., Borges, V.M., Prates, D.B., de Souza, A.P., Barral, A., Balanco, J.M., do Nascimento, M.T., Saraiva, E.M. and Barcinski, M.A. (2009). Cooperation between apoptotic and viable metacyclics enhances the pathogenesis of Leishmaniasis. *Plos One 4*, e5733.

Wang, L., Kou, M.C., Weng, C.Y., Hu, L.W., Wang, Y.J. and Wu, M.J. (2012). Arsenic modulates heme oxygenase-1, interleukin-6, and vascular endothelial growth factor expression in endothelial cells: roles of ROS, NF-kB, and MAPK pathways. *Archives of Toxicology 86*, 879-896.

Watanabe, C., Kawata, A., Sudo, N., Sekiyama, M., Inaoka, T., Bae, M. and Ohtsuka, R. (2004). Water intake in an Asian population living in arsenic-contaminated area. *Toxicology and Applied Pharmacology 198*, 272-282.

Welch, A.H., Westjohn, D.B., Helsel, D.R. and Wanty, R.B. (2000). Arsenic in ground water of the United States: Occurrence and geochemistry. *Ground Water 38*, 589-604.

Williams, P., Raab, A., Feldmann, J. and Meharg, A. (2007a). Market basket survey shows elevated levels of as in South Central US processed rice compared to California: Consequences for human dietary exposure. *Environmental Science & Technology 41*, 2178-2183.

Williams, P.N., Villada, A., Deacon, C., Raab, A., Figuerola, J., Green, A.J., Feldmann, J. and Meharg, A.A. (2007b). Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat and barley. *Environmental Science & Technology 41*, 6854-6859.

World Health Organization. (2010). Control of the Leishmaniases. *949*, 1-186.

Wu, M.M., Chiou, H.Y., Ho, I.C., Chen, C.J. and Lee, T.C. (2003). Gene expression of inflammatory molecules in circulating lymphocytes from arsenic-exposed human subjects. *Environmental Health Perspectives 111*, 1429-1438.

Wyllie, S., Cunningham, M.L. and Fairlamb, A.H. (2004). Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. *Journal of Biological Chemistry 279*, 39925-39932.

Wyllie, S. and Fairlamb, A.H. (2006a). Differential toxicity of antimonial compounds and their effects on glutathione homeostasis in a human leukaemia monocyte cell line. *Biochemical Pharmacology 71*, 257-267.

Wyllie, S. and Fairlamb, A.H. (2006b). Refinement of techniques for the propagation of *Leishmania donovani* in hamsters. *Acta Tropica 97*, 364-369.

Wyllie, S., Mandal, G., Singh, N., Sundar, S., Fairlamb, A.H. and Chatterjee, M. (2010). Elevated levels of tryparedoxin peroxidase in antimony unresponsive *Leishmania donovani* field isolates. *Molecular and Biochemical Parasitology 173*, 162-164.

Wyllie, S., Patterson, S., Stojanovski, L., Simeons, F.R., Norval, S., Kime, R., Read, K.D. and Fairlamb, A.H. (2012). The anti-trypanosome drug fexinidazole shows potential for treating visceral leishmaniasis. *Sci. Transl. Med 4*, 119re1.

Wyllie, S., Vickers, T.J. and Fairlamb, A.H. (2008). Roles of trypanothione *S*-transferase and tryparedoxin peroxidase in resistance to antimonials. *Antimicrobial Agents and Chemotherapy 52*, 1359-1365.

Xu, Y., Li, X., Zheng, Q., Wang, H., Wang, Y. and Sun, G. (2009). Lack of association of glutathione-S-transferase omega 1(A140D) and omega 2 (N142D) gene polymorphisms with urinary arsenic profile and oxidative stress status in arsenic-exposed population. *Mutation Research 679*, 44-49.

Yan, S., Jin, L., and Sun, H. (2005). $_{51}$Sb Antimony in Medicine. In *Metallotherapeutic Drugs & Metal-based Diagnostic Agents, The use of metals in medicine*, M. Gielen and E.R.T. Tiekink, eds., pp. 441-461.

Yan, S.C., Li, F., Ding, K.Y. and Sun, H.Z. (2003). Reduction of pentavalent antimony by trypanothione and formation of a binary and ternary complex of antimony(III) and trypanothione. *Journal of Biological Inorganic Chemistry 8*, 689-697.

Yardley, V., Ortuno, N., Llanos-Cuentas, A., Chappuis, F., Doncker, S.D., Ramirez, L., Croft, S., Arevalo, J., Adaui, V., Bermudez, H., Decuypere, S. and Dujardin, J.C. (2006). American tegumentary leishmaniasis: Is antimonial treatment outcome related to parasite drug susceptibility? *Journal of Infectious Diseases 194*, 1168-1175.

Yuan, Y., Marshall, G., Ferreccio, C., Steinmaus, C., Selvin, S., Liaw, J., Bates, M.N. and Smith, A.H. (2007). Acute myocardial infarction mortality in comparison with lung and bladder cancer mortality in arsenic-exposed region II of Chile from 1950 to 2000. *Am. J. Epidemiol. 166*, 1381-1391.

Zhang, C., Yao, H. and Ling, B. (1998). [Accumulative arsenic level in viscera in offsprings of exposed parent rats]. *Zhonghua Yu Fang Yi. Xue. Za Zhi. 32*, 76-78.

Zhou, Y., Bhattacharjee, H. and Mukhopadhyay, R. (2006). Bifunctional role of the leishmanial antimonate reductase LmACR2 as a protein tyrosine phosphatase. *Molecular and Biochemical Parasitology 148*, 161-168.

Zhou, Y., Messier, N., Ouellette, M., Rosen, B.P. and Mukhopadhyay, R. (2004). *Leishmania major* LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug Pentostam. *Journal of Biological Chemistry 279*, 37445-37451.

Zijlstra, E.E., El-Hassan, A.M. and Ismael, A. (1995). Endemic kala-azar in eastern Sudan: post-kala-azar dermal leishmaniasis. *American Journal of Tropical Medicine and Hygiene 52*, 299-305.